昭和 32 年 7 月 9 日 第三種郵便物認可
第46巻第 5 号 昭和34年 5 月25日発行
(毎月 1 回 25 日発行)
The Journal of Biochemistry, published monthly by the Japanese Biochemical Society, Tokyo, Japan.
Vol. 46, No. 5, May 25, 1959

Vol. 46, No. 5 **May, 1959**

THE JOURNAL

OF

BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

PUBLISHED MONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle Rutlant, Vt. U.S.A.
Maruzen Co., Ltd. Tokyo Japan
Santo Vanasio Köln Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Departments of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be markedly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts, besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units, and periodicals will be sent immediately.

Subscription

Subscription Price. ¥3,600 (U.S. $10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥3,600 (U.S. $10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University Bunkyo-hu, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co., Ltd.*, Chuo-ku, Tokyo, Japan.

The Journal of Biochemistry, Vol. 46, No. 5, 1959

STATIONARY AND NON-STATIONARY STATES OF METABOLIZING SYSTEMS WITH CYCLIC PROCESSES

By TAMITARŌ MIZUNOYA

(From the Laboratory of Biochemical Physics, the Faculty of Agriculture, Kyushu University, Fukuoka)

(Received for publication, March 27, 1958)

The metabolic pathways may be thought to be characterized by the roundabout processes with many intermediate steps. Among these pathways there are many cyclic processes, *e.g.* tricarboxylic acid-, dicarboxylic acid-, fatty acid-, ornithine-arginine-, hexose monophosphate-, succinate-glycine-, and ortho-quinone amino acid cycles *etc.* The biological role of cyclic processes was discussed by H. A. Krebs (*1*). J. Z. Hearon has studied the rate behavior of metabolizing systems and noted that a system, in which a catalyst or a conserved component is present, is necessarily accompanied by a cycle (*2*). Although he also discussed in another paper (*3*) the role of cyclic catalysts in the rates and energetics of cyclic systems, no explicit expression was given for the characteristics of cyclic processes.

The kinetic processes of metabolic reactions are different from the usual kinetics in the point that the system of reactions is not "closed" but "open". Thus the system of metabolic reactions, *i.e.* the metabolizing system, may be physically regarded as a kind of an open reaction system. For the analyses of these systems and the others, the kinetic studies of open reaction systems have been developed by several workers (*4*) along with the studies of thermodynamics of irreversible processes by Prigogine (*5*) and de Groot (*6*) *etc.* According to these theories, the reaction processes in an open system become possible for a stationary or steady state to develop, which however is not a true equilibrium. The real vital processes in the metabolizing systems also proceed in the stationary states with some exceptions. The constancy of the sugar content of blood is a good example of the stationary state.

Then it is the purpose of this paper to elucidate the conditions which make possible for the metabolizing system with cyclic processes to approach to the stationary state. First, the theory of the thermodynamics of irreversible processes is introduced into the general metabolizing systems and then an equation for the rate of dissipation of the available energy in the system is derived. Secondly, the equation is applied to the metabolizing systems with the cyclic processes. It is shown that one of the conditions under which the stationary state appears is the conservation of the amount of cycle components. The condition is realized in both cases: (A) any branch reaction involving the cycle components does not occur; (B) two or more branch reactions involving the cycle components compensate with each other.

Thirdly, the rate of dissipation of the available energy is connected with the velocity of cyclic reaction in the case of (A). Finally, from this equation a suggestion is given to a solution of the problem of the evolution of catalytic chemical cycles in the metabolizing systems. In the case of (B), we can find an example in the succeeding paper (*7*).

PART I. GENERAL THEORY

1. *Energy Dissipation in a Metabolizing System in General.* The metabolizing system may be considered thermodynamically as a sort of an open system as stated above. In other words, the metabolizing system is able to exchange both energy (heat and work) and matter with its environment across the boundary. Thus the metabolizing system and its environment interact with each other. This interrelationship can not be treated by the theory of a closed system of the classical thermodynamics. Terms for the exchange of matter should be added to the balance equations for energy and entropy. After the equations derived by Tolman and Fine (*8*), the changes in the energy ΔU and the entropy ΔS of the metabolizing system are expressed as follows:

$$\Delta U = \sum_m U_m + Q - W, \tag{1}$$

$$\Delta S = \sum_m S_m + \frac{Q}{T} + \Delta S_{\mathrm{irr}}, \tag{2}$$

where $\sum_m U_m$ and $\sum_m S_m$ are the changes in the energy and the entropy due to the exchange of matter with the environment respectively, Q is the heat absorbed reversibly from the environment at a constant temperature T preqailed through both the system and its environment, W is the work done by the system to the environment, and ΔS_{irr} is the entropy increment due to the irreversible processes taking place within the system.

The change in the Gibbs' free energy ΔG of this metabolizing system is given by

$$\Delta G = \Delta U - T\Delta S + p\Delta V, \tag{3}$$

where ΔV is the change of the volume of this system under the common constant pressure p through both the system and its environment. Using Eqs. (1) and (2), it may be rewritten as follows:

$$\begin{aligned} \Delta G &= \sum_m F_m - W - T\Delta S_{\mathrm{irr}} + p\Delta V \\ &= \sum_m G_m - W - T\Delta S_{\mathrm{irr}} \\ \text{or} \quad T\Delta S_{\mathrm{irr}} &= \sum_m G_m - W - \Delta G = -(\Delta G^* + \Delta G), \end{aligned} \tag{4}$$

where $\sum_m F_m$ and $\sum_m G_m$ are the changes in the Helmholtz' and the Gibbs' free energies of the system due to the exchange of matter respectively and $-(\sum_m G_m - W) = \Delta G^*$ is the change in the Gibbs' free energy of the environment. The star (*) represents "of or in the environment" and the same rule applies to the following notations.

If we denote by τ the time required for such changes of the state variables of the metabolizing system as mentioned above, Eq. (4) becomes

$$T\int_t^{t+\tau} \dot{S}\,dt = -\int_t^{t+\tau} (\dot{G}+\dot{G}^*)dt, \tag{5}$$

where the upper dot represents differentiation with time. Although thermodynamical functions are originally defined in relation to an equilibrium state, the relations among these functions are assumed to be valid even in non-equilibrium state. This is one of the fundamental assumptions in the "thermodynamics of irreversible processes". Thus the system is not necessarily in equilibrium states at time t and $t+\tau$. From Eq. (5), it follows that the entropy production σ per unit time is given by

$$T\sigma \equiv T\,\dot{S} = -(\dot{G}+\dot{G}^*). \tag{6}$$

$T\sigma$ has the dimension of energy per time, corresponding to the rate of dissipation of the available energy. This is called the "energy dissipation" in the following parts.

Introducing the chemical potential μ_m of species m, defined as $\mu_m = \partial G/\partial[m]$, where $[m]$ is the concentration of species m, we may rewrite the rate equation (6) of energy dissipation as follows:

$$T\sigma = -\sum_m \left(\mu_m \frac{d[m]}{dt} + \mu_m{}^* \frac{d[m^*]}{dt}\right).^{1)} \tag{7}$$

2. *Formulations of Phenomenological Relations in the Metabolizing System with Cyclic Processes.* The tricarboxylic acid (TCA) cycle may be regarded as a catalytic system which catalyzes a complete oxidation of pyruvic acid to carbon dioxide and water, and this catalytic reaction occurs in the multistep cyclic process. Such a catalytic cycle is expressed as follows:

$$\begin{aligned} &S_1 + C_1 \rightarrow C_2 + P_1,\\ &S_2 + C_2 \rightarrow C_3 + P_2,\\ &\cdots\cdots\cdots\cdots,\\ &S_n + C_n \rightarrow C_1 + P_n,\\ \hline &\sum_{i=1}^{n} S_i \rightarrow \sum_{i=1}^{n} P_i, \end{aligned}$$

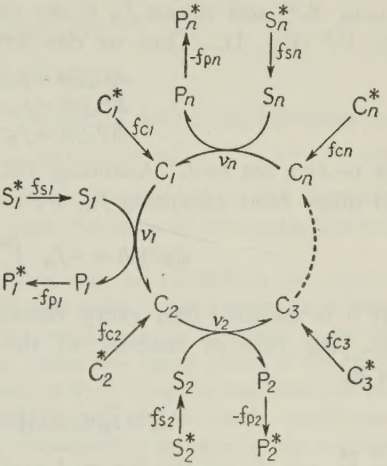


FIG. 1. A metabolizing system with a cyclic process catalyzing the overall reaction from $\sum_i S_i$ to $\sum_i P_i$.

1) This equation has been already derived by K. G. Denbigh (9).

where C_i, S_i and P_i are the member of the catalytic cycle, the substrate and the product of the i-th step respectively (Fig. 1). The overall reaction from $\sum S_i$ to $\sum P_i$ is catalyzed by the cycle consisting of n members C_i's ($i=1, 2...n$). This scheme is applicable not only to such a metabolic pathway as TCA cycle but also to an enzyme reaction which involves two or more enzyme-substrate complexes and catalyzes the overall reaction from $\sum S_i$ to $\sum P_i$ *in vivo*.

With some exceptions, the metabolic flow is generally considered to be stationary. Actually the enzymes involved in the TCA cycle operate at the trace level of the substrates and in the course of the operation no intermediates accumulate in any measurable amount (*10*); in other words, this catalytic reaction proceeds in the stationary state. Therefore, it is interesting to examine from the thermodynamic and kinetic viewpoints, the rate behavior in the stationary state of a catalytic reaction with n-step cyclic process as represented in Fig. 1. At first we will consider the rates of individual processes.

Let us consider the metabolizing system in which a catalytic reaction as shown in Fig. 1 is taking place. Let v_i be the rate of reaction from C_i to C_{i+1} ($i+1=1$ for $i=n$), and f_m be the rate of transfer of species m ($m=C_i$, S_i and P_i), respectively, defined as the change of the concentrations of C_i and m per unit time. If the transfer is spatial, f_m is the rate of permeation of species m from the environment into the system. In this case, according to the above-mentioned rule, m^* represents the species m in the environment. If the transfer is chemical, m^* may be thought to be the precursor of species m. Thus f_{ci} is the rate of formation of C_i from the precursor C_i^*, which is not one of the cycle components, f_{si} the rate of formation of S_i from the precursor S_i^* and minus f_{pi} is the rate of transformation of P_i to another species P_i^* (Fig. 1). Thus we can write as

$$d[C_i]/dt = f_{ci} + v_{i-1} - v_i, \tag{8 a}$$
$$d[S_i]/dt = f_{si} - v_i, \tag{8 b}$$
$$d[P_i]/dt = f_{pi} + v_i, \tag{8 c}$$

where $i-1=n$ for $i=1$. Assuming that the concentration $[m^*]$ is not affected by all other rates excepting f_m, we can write

$$d[m^*]/dt = -f_m \quad \begin{pmatrix} m = C_i,\ S_i \text{ and } P_i \\ i = 1,\ 2,\ldots\ldots n \end{pmatrix}. \tag{9}$$

If it is assumed that every reaction is first order in respect to each species, the rate of reaction of the i-th step, after Prigogine (*5*), is given by

$$v_i = \vec{k}_i[S_i]\,[C_i]\{1-\exp(-A_i/RT)\},^{2)} \tag{10}$$

where $\vec{k}_i$ and A_i are the forward rate constant and the chemical affinity of the reaction of the i-th step respectively and R is the gas constant. The Eyring's theory gives the following expression to $\vec{k}_i$ (*11*):

2) Exactly, we have to use not the concentration but the activity.

$$\vec{k_i} = \frac{kT}{h}\exp(-\vec{\Delta G_i}^*/RT), \tag{11}$$

where k and h are the Boltzman and Planck constants respectively and $\vec{\Delta G_i}^*$ is the standard free energy of activation of the forward reaction. According to the definition introduced by de Donder, the chemical affinity A_i is written as

$$A_i = \mu_{ci} + \mu_{si} - \mu_{ci+1} - \mu_{pi} \quad \begin{pmatrix} i = 1, 2, \ldots\ldots n \\ i+1 = 1 \text{ for } i = n \end{pmatrix}. \tag{12}$$

If the reaction proceeds in nearly equilibrium state, *i.e.* when $A_i \ll RT$,[3] Eq. (10) reduces to

$$v_i = \vec{k_i}[S_i]\,[C_i]A_i/RT. \tag{13 a}$$

Similarly if $A_m \ll RT$ in the chemical transfer of species m, the following is obtained:

$$f_m = \vec{k_m}[m^*]A_m/RT, \tag{14 a}$$

where $\vec{k_m}$ and A_m have similar meanings to the above and

$$A_m = \mu_m{}^* - \mu_m. \tag{15}$$

In real cases, the chemical reactions *in vivo* may be thought to interfere considerably with one another. Therefore, Eqs. (13 a) and (14 a) should be rewritten as

$$v_i = \sum_j L_{ij}A_j + \sum_m L_{im}A_m, \tag{13 b}$$

$$f_m = \sum_i L_{mi}A_i + \sum_l L_{ml}A_l, \tag{14 b}$$

where

$$L_{ii} = \vec{k_i}[S_i]\,[C_i]/RT,^4$$

$$L_{mm} = \vec{k_m}[m^*]/RT,^4$$

and it is expected that there are the Onsager's reciprocal relations, *i.e.*

$$L_{ij} = L_{ji},\ L_{im} = L_{mi} \text{ and } L_{ml} = L_{lm}, \tag{16 a}$$

among the interference coefficients[5], which are related to the interrelationships between the reactions of the i-th and j-th steps of the cycle, the reaction

3) As stated by Prigogine, this assumption may be satisfied in the metabolizing system, where the metabolic reactions usually take place in many intermediate steps, each of which is nearly reversible. This practical reversibility may be attributed to the coupling of exergonic reaction with endergonic one, where the pump action of the energy-rich phosphate bond cycle plays an important role, as Prof. Sugita had discussed (*12*).

4) From the assumption that the reactions proceed in nearly equilibrium, the concentrations $[S_i]$, $[C_i]$ and $[m^*]$ may be considered to be equal to the corresponding one in the equilibrium state respectively and then the coefficients L_{ii} and L_{mm} to be constant. Exactly, these coefficients are not necessarily constant.

5) Both problems are necessary to be examined in future: (a) What are the forms of the formulae defining the interference coefficients? (b) Are there in truth the reciprocal relations among these coefficient?

of the i-th step and the chemical transfer of species m, and the chemical transfers of species m and l, respectively.

If the transfer is spatial, the rate f is vectorial and can not be coupled with the rate of chemical reaction that is scalar. Hence it follows that

$$v_i = \sum_j L_{ij} A_j, \tag{13 c}$$

$$f_m = \sum_l L_{ml} A_l. \tag{14 c}$$

The latter equation is derived from the generalized Fick's law. L_{mm} is the conductance (*i.e.* the inverse of the resistance) of the permeation of species m and L_{ml} $(m \neq l)$ is the coefficient related to the interrelationships between the permeations of speies m and l. In this case, we also expect the Onsager's relations

$$L_{ij} = L_{ji} \text{ and } L_{ml} = L_{lm}. \tag{16 b}$$

3. *Stationary State of the Metabolizing System with Cyclic Processes.* In view of Eqs. (7), (8), (9), (12) and (15), the metabolizing system considered in the previous section dissipates the available energy at the following rate:

$$T\sigma = \sum_i v_i A_i + \sum_m f_m A_m. \tag{17}$$

Inserting Eqs. (13) and (14) into Eq. (17), we obtains a quadratic function of the chemical affinities, which is positive definite. Therefore the following relations are found:

$$\begin{aligned} &L_{ii} \text{ and } L_{mm} \geqslant 0, \\ &L_{ii}^2 - L_{ij} L_{ji} \geqslant 0, \\ &L_{mm}^2 - L_{ml} L_{lm} \geqslant 0, \text{ etc.} \end{aligned} \tag{18}$$

If we consider the equations from (12) to (15) and the relation, $\mu_m = \mu_m^0 + RT \ln \alpha_m [m]$, where α_m is the activity coefficient of species m, it is clear that $T\sigma$ is prescribed by the independent variables μ_m's and μ_m*'s. The circumstances that μ_m* is constant may be realized by the homeostatic character of organism due to the complicated controlling mechanisms. If the chemical potential μ_m* is kept constant, the condition for the minimum entropy production is given by

$$\partial\sigma/\partial\mu_m = 0 \quad (m = \mathrm{C}_i, \mathrm{S}_i \text{ and } \mathrm{P}_i). \tag{19}$$

In this differentiation, all other chemical potentials than μ_m have to be kept constant. The state of the minimum entropy production is called the stationary state by Prigogine (*5*) and de Groot (*6*). Applying the differentiation (19) to the quadratic function mentioned above and using the Onsager's relations (16), we may find

$$\begin{aligned} &T\partial\sigma/\partial\mu_{\mathrm{c}i} = 2(v_i - v_{i-1} - f_{\mathrm{c}i}) = 0, \\ &T\partial\sigma/\partial\mu_{\mathrm{s}i} = 2(v_i - f_{\mathrm{s}i}) = 0, \\ &T\partial\sigma/\partial\mu_{\mathrm{p}i} = -2(v_i + f_{\mathrm{p}i}) = 0. \end{aligned} \tag{20}$$

Comparing Eqs. (20) with Eq. (8) it follows that

$$\frac{d[\mathrm{C}_i]}{dt} = 0, \quad \frac{d[\mathrm{S}_i]}{dt} = 0 \text{ and } \frac{d[\mathrm{P}_i]}{dt} = 0. \tag{21}$$

These relations show that the concentration of each species in the metabo-

lizing system does not change with time. This is the stationary state introduced by Bertalanffy (*4 a*). Thus only when the linear relations (13) and (14) and the Onsager's relations (16) are satisfied, the Prigogine's stationary state is identical with the Bertalanffy's one[6].

From Eqs. (20), the relations among the rates in the stationary state are obtained as follows:

$$v_i = v_{i-1} + f_{ci} = f_{si} = -f_{pi} \quad \begin{pmatrix} i = 1, 2, \ldots\ldots, n \\ i-1 = n \text{ for } i = 1 \end{pmatrix}, \tag{22}$$

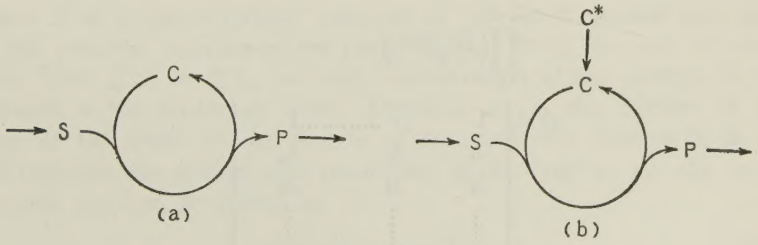
$$\sum_{i=1}^{n} f_{ci} = 0. \tag{23}$$

Thus the reaction of catalytic cycle can proceed in the stationary state only when the sum of the transfer rates of the catalyst or cycle components is zero, that is, when the total concentration of the catalyst or cycle components is kept constant. If the total concentration of the catalyst is able to change by any reaction, the change should be compensated by other reactions for the maintenance of the stationary state. Such a phenomenon may be found in the contribution of the Wood-Werkman reaction to the TCA cycle, which will be discussed in the succeeding paper by the author (*7*). In the following part of this paper, a special case will be discussed in which any branch reaction involving the cycle components does not take place so that the amount of cyclic catalyst is kept constant.

PART II. A SPECIAL CASE IN WHICH CATALYTIC CYCLE IS "CLOSED"

1. *Description of the System of Closed Catalytic Cycle.* The relation among the rates in the stationary state of the system of catalytic cycle was given by Eqs. (22) and (23). If all f_{ci} are independent of one another, the relation (23) is satisfied under the condition that all f_{ci} are zero. This condition means that no reactions or transfers take place, which are able to vary the amount of the catalyst or cycle conponents. Let such a catalytic cycle be called a "closed catalytic cycle" (Fig. 2).

In order to focus the view to the catalytic cycle, let the substrates and



A Closed Catalytic Cycle An Open Catalytic Cycle

FIG. 2

6) See references (*9*) and (*13*).

the products be freely permeable across the boundary between the system and its environment. In this case, the concentrations or the chemical potentials of each substrate and product within the system are respectively equal to the corresponding one in the environment. We may suppose the circumstances in which the reactions taking place in the environment supply the substrates into the system and remove the products from the system so that the concentrations of each substrate and product are kept constant at a finite value and practically at zero, respectively. That is

$$\begin{aligned} &[S_i] = [S_i^*] = \text{constant and } [P_i] = [P_i^*] \approx 0 \\ \text{or}\quad &\mu_{si} = \mu_{si}^* = \text{constant and } \mu_{pi} = \mu_{pi}^* = \text{constant.} \end{aligned} \tag{24}$$

These circumstances satisfy the assumption under which the condition (19) for the stationary state is deduced. Under these circumstances the reverse reaction in each step of the catalytic cycle may be neglected and the concentration of substrate can be put into the rate constant as follows:

$$v_i = k_i[C_i] \text{ where } k_i = \overrightarrow{k_i}[S_i]. \tag{25}$$

2. *Kinetics of Closed Catalytic Cycle.* Under the circumstances described in the previous section, Eqs. (8 a), (8 b) and (8 c) reduce to

$$d[C_i]/dt = k_{i-1}[C_{i-1}] - k_i[C_i], \tag{26 a}$$
$$d[S_i]/dt = 0, \tag{26 b}$$
$$d[P_i]/dt = 0, \tag{26 c}$$

where $i-1=n$ for $i=1$. From the assumption that all f_{ci} are zero, the total concentration of the catalyst or cycle components is kept at a constant value $[C_0]$. It is assumed that the total amount of the catalyst or cycle components occupies the state of the first member at the initial moment, *i.e.*

$$[C_1] = [C_0] \text{ and } [C_2] = [C_3] = \cdots\cdots = [C_n] = 0 \text{ at } t = 0. \tag{27}$$

Under this initial condition, the general solution for Eq. (26 a) is given by

$$[C_i] = \sum_{j=1}^{n} \left\{ \prod_{i}^{n-1} \left(\frac{\lambda_j + k_{i+1}}{k_i} \right) (-1)^{n+j} k_1 k_2 \ldots k_{n-1} [C_0] \frac{D_{\lambda_j^{n-1}}}{D} e^{\lambda_j t} \right\}, \tag{28}$$

where

$$\prod_{i}^{n-1} \left(\frac{\lambda_j + k_{i+1}}{k_i} \right) = 1 \text{ for } i = n,$$

$$D = \begin{vmatrix} 1 & 1 & \cdots\cdots & 1 \\ \lambda_1 & \lambda_2 & & \lambda_n \\ \lambda_1^2 & \lambda_2^2 & & \lambda_n^2 \\ \vdots & \vdots & & \vdots \\ \lambda_1^{n-1} & \lambda_2^{n-1} & \cdots\cdots & \lambda_n^{n-1} \end{vmatrix},$$

and $D_{\lambda_j^{n-1}}$ is the minor determinant of the element λ_j^{n-1}. There are the following relations among λ_j's ($j=1, 2, \ldots, n$) and k_i's ($i=1, 2, \ldots, n$):

$$\lambda_1 = 0,\ \sum_{j=2}^{n}\lambda_j = -\sum_{i=1}^{n}k_i,\ \sum_{j=2}^{n}\lambda_2\lambda_3 = \sum_{i=1}^{n}k_1k_2,$$
$$\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots,$$
$$\lambda_2\lambda_3\cdots\cdots\lambda_n = (-1)^{n-1}\sum_{i=1}^{n}k_1k_2\cdots\cdots k_{n-1}. \tag{29}$$

Inserting $\lambda_1=0$ into Eq. (28) we obtains

$$[C_i] = \prod_{i}^{n-1}\left(\frac{k_{i+1}}{k_i}\right)(-1)^{n+1}k_1k_2\cdots\cdots k_{n-1}[C_0]\frac{D_{\lambda_1^{n-1}}}{D}$$
$$+\sum_{j=2}^{n}\left\{\prod_{i}^{n-1}\left(\frac{\lambda_j+k_{i+1}}{k_i}\right)(-1)^{n+j}k_1k_2\cdots\cdots k_{n-1}[C_0]\frac{D_{\lambda_j^{n-1}}}{D}e^{\lambda_j t}\right\}. \tag{30}$$

The first term is time-independent, *i.e.* the stationary term, while the second term is time-dependent, *i.e.* the non-stationary term. From the relations (29), all the other λ_j's than λ_1 are negative, because all k_i's are positive. Hence, the non-stationary term is vanished at $t=\infty$, that is, the concentration $[C_i]$ approaches to the stationary value $[C_i^0]$, which is written as

$$[C_i^0] = [C_0]/k_i\sum_{i=1}^{n}(1/k_i). \tag{31}$$

Putting this expression into Eq. (25) we obtains the rate of reaction of the i-th step in the stationary state as follows:

$$v_i^0 = k_i[C_i^0] = [C_0]/\sum_{i=1}^{n}(1/k_i) = \mathfrak{k}[C_0], \tag{32}$$

where

$$\mathfrak{k} \equiv 1/\sum_{i=1}^{n}\left(\frac{1}{k_i}\right) = v_i^0/[C_0]. \tag{33}$$

This shows that the reaction rate of each step of the cycle is equal with one another, that is, has a common value independent of the steps of cyclic process. This fact can easily be seen by putting $f_{ci}=0$ in Eq. (22) as follows:

$$v_i^0 = v_{i-1}^0 \quad \begin{pmatrix} i = 1,\ 2,\cdots\cdots n \\ i-1 = n \text{ for } i = 1 \end{pmatrix}. \tag{34}$$

Therefore $\mathfrak{k}$ is a characteristic constant of the cyclic process and can be called the turnover number of the cycle because it is the rate of overall reaction from ΣS_i to ΣP_i per unit concentration of the catalyst or cycle components in the stationary state. Denoting by τ_i the lifetime of each member of the cycle or the inverse of apparent rate constant k_i of each step of reaction, the period of a revolution of the cycle τ, *i.e.* the inverse of turnover number, is written as

$$\tau \equiv 1/\mathfrak{k} = \sum_{i=1}^{n}(1/k_i) = \sum_{i=1}^{n}\tau_i. \tag{35}$$

If the rate of overall reaction is determined by the reaction rate of the j-th step, that is, if k_j is much smaller than the others, Eqs. (31), (32), (33) and (35) respectively reduce to

$$[C_i^0] = \frac{k_j}{k_i}[C_0], \quad v_i^0 = k_j[C_0], \text{ and } 1/\tau \equiv \mathfrak{k} = k_j. \tag{36}$$

3. *Energy Dissipation in the Stationary State.* In the previous section, it was shown that the transiton among the catalyst or cycle components becomes a stationary vortex (or circulation) under the conditions that all f_{ci} are zero and that the chemical potentials μ_{si}^* and μ_{pi}^* are constant. Denoting by v^0 the volocity of the stationary vortex and using Eq. (17), the energy dissipation $T\sigma^0$ in this stationary state is written as

$$T\sigma^0 = v^0 X, \tag{37}$$

where

$$v^0 \equiv v_i^0 = v_{i-1}^0 = f_{si}^0 = -f_{pi}^0, \tag{38}$$

$$X \equiv \sum_i (\mu_{si}^* - \mu_{pi}^*). \tag{39}$$

X is the driving force for the stationary vortex of the catalyst or cycle components, that is the chemical affinity of the overall reaction from $\sum S_i$ to $\sum P_i$. Using Eq. (32) for the velocity v^0, the equation of energy dissipation (37) becomes

$$T\sigma^0 = [C_0]X \Big/ \sum_{i=1}^{n} \left(\frac{1}{k_i}\right) = [C_0]\, X \Big/ \sum_{i=1}^{n} \tau_i. \tag{40}$$

If the reaction of the j-th step is rate-determining, Eq. (40) reduces to

$$T\sigma^0 = k_j[C_0]X, \tag{41 a}$$

or furthermore, using Eqs. (25) and (11) we obtains

$$T\sigma^0 = \overrightarrow{k_j}[S_j][C_0]X \tag{41 b}$$

$$= \frac{kT}{h}\exp\left(-\Delta\overrightarrow{G}_j^*/RT\right)[S_j]\,[C_0]\,X. \tag{41 c}$$

The energy dissipation in the stationary reaction of catalytic cycle depends on the total concentration of the catalyst or cycle components and the chemical affinity of the overall reaction, as well as the activation energy and the concentration of substrate for the rate-determining step in the catalytic cycle.

From Eq. (40), the energy dissipation per unit concentration of the catalyst or cycle components in the stationary state is written as

$$T\sigma^0/[C_0] = X \Big/ \sum_{i=1}^{n} \tau_i. \tag{42 a}$$

This equation can also be derived in the following way by another consideration.

The general expression for the energy dissipation in the metabolizing system was given in Section 1 of Part I as

$$T\sigma \equiv T\dot{S} = -(\dot{G} + \dot{G}^*), \tag{6}$$

but if τ in Eq. (5) is so small that there are linear relations among the state variables and time, $T\sigma$ may be approximately given by

$$T\sigma \approx T\Delta S_{irr}/\tau = -(\Delta G + \Delta G^*)/\tau. \tag{43}$$

In the case of stationary or cyclic irreversible processes, the Gibbs' free energy of the system does not change, namely $\Delta G=0$. Hence, it follows that

$$T\sigma \approx -\Delta G^*/\tau = (\sum_m G_m - W)/\tau. \tag{44}$$

If we consider the energy dissipation per unit concentration of species m defined as $T\mathfrak{S}=T\sum_m \partial\sigma/\partial[m]$, it is derived from the definition of the chemical potential and Eq. (44) as follows

$$T\mathfrak{S} = \sum_m \mu_m/\tau = \sum_i (\mu^*_{si}-\mu^*_{pi})\Big/\sum_i \tau_i = X\Big/\sum_i \tau_i, \tag{42 b}$$

where Σ_m is the sum over all species exchanged between the metabolizing system and its environment and Σ_i is the sum over all reaction steps of closed catalytic cycle. This equation is completely equivalent to Eq. (42 a).

4. *Suggestions to the Evolution of the Catalytic Cycles in the Metabolizing Systems.* From Eq. (40) or (42) it is clear that the energy dissipation is inversely proportional to the period of a revolution of the cycle. If the facts are taken into account that biological activities reveal themselves at a constant temperature and are sensitive to heat, it may be said, from the viewpoint of fitness or "Zweckmässigkeit", that the metabolizing system which dissipates the available energy at a less rate than the other is more fitting or "zweckmässiger". In judging which is the more primitive among some metabolizing systems, various factors that influence the evolution have to be taken into consideration. The author proposed the "energy dissipation rate" as a thermodynamical criterion for the evolution of the metabolizing systems, the validity of which was discussed in another paper (*14*). If the adaptative evolution of the metabolizing systems proceeds toward fitness, the metabolizing system which has a higher rate of energy dissipation than the other is considered to be more primitive. Comparing two different systems which have the same value of ΔG^* in Eq. (44) or X in Eq. (40), the system with a shorter period τ dissipates the available energy at a higer rate. According to the above viewpoint, it may be more primitive than the other. If τ_i-values, are quantities of the same order, the number of reaction steps can be used instead of the period τ. In comparing some cyclic systems which catalyze the same overall reaction with one another, it may possibly be said that the system of a roundabout pathway with more multi-steps is more developed than the others and the system of triangle cyclic reactions is the most primitive. In other words, it may be suggested that in the process of biochemical evolution of the catalytic systems, organisms may probably have developed to gain new reaction steps and to complicate the metabolic patterns. An example may be found in the comparison between the tri- and dicarboxylic acid cycles (*14*).

CONCLUSIONS

The theory of the "thermodynamics of irreversible processes" applied

to the metabolizing system with the reactions of catalytic cycle shows that the reactions can proceed in the stationary state only when the total concentration of the catalyst or cycle components is kept constant (Part I).

Such a situation is realized in the following cases: (a) any branch reaction involving the catalyst or cycle components does not take place; (b) two or more branch reactions involving the catalyst or cycle components compensate with each other.

In the former case, the transition among the cycle components becomes the stationary vortex (or circulation). The velocity of the stationary vortex is proportional to the total concentration of the catlyst or cycle components. The proportional coefficient $\tilde{f}$ is the turnover number of the cyclic process, and its inverse, *i.e.* the period of a revolution of the cycle, is expressed as the sum of lifetimes of the cycle components. The rate equation derived from the above results shows that the energy dissipation in the reaction of catalytic cycle is directly proportional to the total concentration of the catalyst or cycle components as well as the chemical affinity of the overall reaction and is inversely proportional to the period of a revolution of the cycle. The equation leads us to a suggestion that the biochemical evolution of the catalytic chemical cycle has probably proceeded towards the direction of a longer period of a revolution of the cycle and resulted in the gain of new reaction steps and the complication of the metabolic patterns (Part II).

SUMMARY

This paper considers some aspects of the stationary state of the metabolizing system which has such a cyclic pathway as the TCA cycle. In Part I, first of all, an equation for the rate of dissipation of the available energy in the metabolizing system is derived, in general, from the viewpoint of the "thermodynamics of irreversible processes". Secondly this equation is applied to the system with a reaction of catalytic cycle and it is shown that the stationary state appears only under the situation of the conservation of the amount of cycle components. In Part II, a special case is kinetically treated in which any branch reaction which is able to vary the amount of the catalyst or cycle components themselves does not occur, and it is shown that the reaction rate of each step in the cyclic process becomes equal with one another as the time goes to infinity; in other words, the stationary vortex appears among the cycle components. Subsequently, the energy dissipation $T\sigma^0$ in the stationary vortex of the cyclic catalyst is formulated as

$$T\sigma^0 = [C_0]\, X/\tau,$$

where $[C_0]$ is the total concentration of the catalyst or cycle components, X is the chemical affinity of the overall reaction catalyzed by the cyclic process and τ is the period of a revolution of the cycle. This equation can be alternatively derived from an approximate equation of the energy

dissipation. Finally, it is suggested from this equation that the catalytic systems with cyclic processes have been probably developed towards the direction of a longer period of the cycle and the metabolic patters have been perhaps complicated by the addition of new reaction steps in the cyclic processes.

The author wishes to express his hearty thanks to Prof. K. Oomori and Ass. Prof. G. Tomita of this laboratory for their advice and encouragement, and to Prof. S. Akabori of Osaka University for encouragement and interest in his work. The authr's thanks are especially due to Prof. M. Sugita of Hitotsubashi University for his valuable advice and continual encouragement and to Prof. K. Watanabe of this university for his kind advice in preparation of the manuscript.

REFERENCES

(*1*) Krebs, H.A., *Enzymologia*, **12**, 88 (1948)
(*2*) Hearon, J.Z., *Physiol. Rev.*, **32**, 499 (1952)
(*3*) Hearon, J.Z., *Bull. Math. Biophys.*, **12**, 57 (1950)
(*4a*) Bertalanffy, L. von, *Naturwiss.*, **28**, 521 (1940)
(*4b*) Bierman, A., *Bull. Math. Biophys.*, **16**, 97 (1954)
(*4c*) Denbigh, K.G., Hicks, M., and Page, F.M., *Trans. Faraday Soc.*, **44**, 479 (1948)
(*4d*) Branson, H., *Arch. Biochem. Biophys.*, **36**, 48 (1952)
(*5*) Prigogine, I., Etude thermodynamique des phénomenès irréversibles, Dunod, Paris (1947)
(*6*) Groot, S. R. de, Thermodynamics of Irreversible Processes, North-Holland Publ. Co., Amsterdam (1951)
(*7*) Mizunoya, T., *J. Biochem.*, in press.
(*8*) Tolman, R.C., and Fine, P.C., *Rev. Mod. Phys.*, **20**, 51 (1948)
(*9*) Denbigh, K.G., *Trans. Faraday Soc.*, **48**, 389 (1952)
(*10*) Green, D.E., Enzymes and Enzyme Systems, ed. by Edsall, J.T., Harvard Univ. Press, Cambridge, p. 28 (1951)
(*11*) Glasstone, S., Laidler, K.J., and Eyring, H., The Theory of Rate Processes, McGraw-Hill Book Co., New York (1941)
(*12*) Sugita, M., *Ann. Hitostubashi Acad.*, **4**, 163 (1954)
(*13*) Bak, T.A., *J. Phys. Chem.*, **59**, 665 (1955)
(*14*) Mizunoya, T., *J. Biochem.*, **46**, 213 (1959)

The Journal of Biochemistry, Vol. 46, No. 5, 1959

STUDIES ON EGG YOLK PROTEINS

VI. ELECTROPHORETIC STUDY OF THE INTERACTION OF β-LIPOVITELLIN WITH POLYANIONS

By HIROSHI SUGANO

(From the Department of Chemistry, Faculty of Science, Niigata University, Niigata)

(Received for publication, June 16, 1958)

Up to the present, numerous investigations have been carried out about the complex formation of protein with nucleic acids (*1-4*), heparin (*5*, *6*), chondroitin sulfate (*6*, *7*), hyaluronic acid (*7*), etc. In these studies, serum albumin has been widely used as a protein material, because it is examined extensively on the physical and chemical properties and has a marked binding ability with small molecules or anions (*8*). It is generally accepted that the nature of the interaction is electrostatic, because the complex formation is inhibited in a medium of higher pH or ionic strength.

Recently, Rosenberg *et al.* (*9*) have found that human serum β-lipoprotein is the only reported example of a protein which binds anions like methyl orange on the alkaline side of the isoelectric point as strongly as serum albumin. Oncley *et al.* (*10*) have reported the use of dextran sulfate as a specific complexing agent for the isolation of β-lipoprotein from serum. Also, Bernfeld *et al.* (*11*) have established by electrophoretic analysis that, in the range of pH 7.5 to 8.6, β-lipoglobulin is the only protein in human serum to interact with certain macromolecular polysulfate esters.

Thus, it seems that lipoproteins have a characteristic high affinity against small anions or large polyanions. In this work, in order to obtain further information on the interactions of lipoprotein with polyanions, the interactions of β-lipovitellin, a purified major lipoprotein of hen's egg yolk (*12*), with nucleic acid, heparin, and alginic acid have been studied at the pH range of alkaline side of the isoelectric point of β-lipovitellin. As the results, it has been represented that β-lipovitellin interacts with these polyanions in the pH range of 7.8 to 9.8. Some informations on the interactions have been described.

EXPERIMENTALS

Materials—The ribonucleic acid (yeast) used was the G.R. grade reagent of T.C.I. (Tokyo Chemical Industries Ltd.). The sodium salt of a commercial preparation of heparin, purchased from Wako Pure Chemical Industries Ltd. (Osaka), with 100 U.S.P. units per mg., was used. The sodium alginate used was a product from Kanto Chemical Co., Inc. (Tokyo). These polyelectrolytes represented a single peak on electrophoresis in

the pH range of 7.8 to 9.8.

The β-lipovitellin was prepared by the method described in the previous paper (*12*). This preparation represented a single peak on electrophoretic analysis in the wide pH range of 4.9 to 10.5, but ultracentrifugation at pH 9.8 showed a heterogeneous pattern (*12*). The β-lipovitellin contained about 40 per cent of alcohol-extractable lipid, almost of which consisted of phospholipid (*12*).

Electrophoresis—Electrophoresis was carried out in a Tiselius apparatus with a cylindrical lens-diagonal slit optical system. Macro cell (10 ml.) was used through all experiments. The carbonate (pH 9.8), ammonium (pH 9.8), and phosphate (pH 7.8) buffers were used. The ionic strength of the buffers was raised over 0.1 by adding NaCl. The pH of the buffers was checked by Beckman Model G instrument. The protein concentration was determined refractometrically. The specific conductivity of the dialyzed buffer was measured at 0°.

Addition of Polyelectrolytes—The desired concentrations of protein and polyanions were obtained as follows; to 10 ml. of the polyelectrolyte solution having twice the final concentration was added 10 ml. of the protein solution having twice the final concentration. The mixture was dialyzed against the buffer for two days by using cellophane bag in a cold room before electrophoresis. The dialysate was always used to fill the upper parts of the electrophoresis cell and the electrode vessels.

RESULTS

Electrophoresis of the Mixture of β-Lipovitellin and Ribonucleic Acid (RNA)—The results of electrophoresis of the mixture of β-lipovitellin and RNA are shown in Table I and Fig. 1-a. In carbonate buffer of pH 9.8 and μ 0.1, an addition of small amount of RNA caused no alteration of the mobility of β-lipovitellin, but at a high RNA concentration, an increase in the mobility was found in the ascending boundary alone. A similar increase in the ascending mobility of β-lipovitellin was also in ammonium buffer of

Table I

Electrophoretic Results of the Mixture of β-Lipovitellin (β-Lv) and RNA

Buffers		Composition (*mg./10 ml.*)		Mobilities (10^{-5} $cm^2 . sec^{-1} . volt^{-1}$)			
				As.		Des.	
pH	μ	β-Lv	RNA	Fast	Slow	Fast	Slow
9.8	0.1[1]	80			3.3		3.1
9.8	0.1[1]		100	17.1		14.1	
9.8	0.1[1]	80	100	17.1	5.0	14.1	3.4
9.8	0.1[2]	80			5.7		5.3
9.8	0.1[2]	80	33	16.3	5.7	15.5	5.1
9.8	0.1[2]	80	135	16.9	6.35	15.2	5.25
7.8	0.25	80			1.25		1.2
7.8	0.25	80	33	12.8	1.55	12.1	1.4

1) Ammonium buffer. 2) Carbonate buffer.

pH 9.8 and μ 0.1. In phosphate buffer of pH 7.8 and μ0.25*, an appreciable increase in the ascending mobility, with a minute increase in the descending mobility, of β-lipovitellin was recognized even at a low RNA concentration.

Electrophoresis of the Mixture of β-Lipovitellin and Heparin—The results of electrophoresis on the system of β-lipovitellin and heparin are shown in Table II and Fig. 1-b. A marked increase in the ascending mobility of β-lipovitellin was found in carbonate buffer of pH 9.8 and μ 0.1. This increase did not disappear though the ionic strength was raised up to 0.3. In contrast to the changes observed in the ascending mobility, there were no changes evident in the corresponding descending mobility of β-lipovitellin in both cases. In phosphate buffer of pH 7.8 and 0.3, a considerable amount of precipitate was formed during dialysis**. Electrophoresis on the supernatant solution represented not only a marked increase in the ascending mobility but also an appreciable increase in the descending mobility of β-lipovitellin.

TABLE II

Electrophoretic Results of the Mixture of β-Lv and Heparin

Buffers		Composition (*mg./10 ml.*)		Mobilities (10^{-5} cm^2. sec^{-1}. $volt^{-1}$) As.		Des.	
pH	μ	β-Lv	Heparin	Fast	Slow	Fast	Slow
9.8	0.1	80			5.7		5.3
9.8	0.1		33	18.8		17.4	
9.8	0.1	80	33	18.3	7.8	16.2	5.5
9.8	0.3	80			3.5		3.5
9.8	0.3	80	33	13.9	4.5	13.2	3.9
7.8	0.25	80			1.25		1.2
7.8	0.3[1)]	80	33	13.6	3.35	13.0	2.05

1) Electrophoretic analysis was performed with the supernatant of the precipitate produced during the dialysis.

Electrophoresis of the Mixture of β-Lipovitellin and Alginic Acid—The results of electrophoresis on the system of β-lipovitellin and alginic acid are shown in Table III and Fig. 1-c, d, e. Like the above two cases, a marked increase in the ascending mobility of β-lipovitellin was observed in carbonate buffer of pH 9.8 and μ 0.1. This increase was rather inhibited by raising the ionic strength up to 0.3. In contrast to the ascending mobilities, the corresponding descending mobilities of β-lipovitellin exhibited no major changes at all alginic acid concentrations. A marked increase in the ascending mobility, with an appreciable increase in the descending mobilty, of β-lipovitellin

* At pH 7.8, a partial precipitation of β-lipovitellin occurred at the ionic strength below 0.25 (*12*).

** This precipitate dissolved again by raising the pH of medium.

TABLE III

Electrophoretic Results of the Mixture of β-Lv and Alginic Acid

Buffers		Composition		Mobilities (10^{-5} cm^2. sec^{-1}. $volt^{-1}$)				Relative area[1]			
		(*mg./10 ml.*)		As.		Des.		As.		Des.	
pH	μ	β-Lv	Alginic acid	Fast	Slow	Fast	Slow	Fast	Slow	Fast	Slow
9.8	0.1	80			5.7		5.3				
9.8	0.1	80	6.7	15.8	6.95	14.6	5.3	5.8	158	21.9	148
9.8	0.1		6.7	16.5		15.7					
9.8	0.1	80	20	16.3	8.95	14.3	5.2	18.5	141	62	112
9.8	0.1		20	16.8		15.1					
9.8	0.1	80	33	16.8	11.0	14.0	4.9	31.4	135	104	66
9.8	0.1		33	17.0		14.2					
9.8	0.3	80	33	11.3	5.45	10.3	3.8				
9.8	0.3	80			3.5		3.5				
7.8	0.25	80	33	12.3	3.75	11.7	1.95				
7.8	0.25	80			1·25		1.2				

1) The appropriate unit.

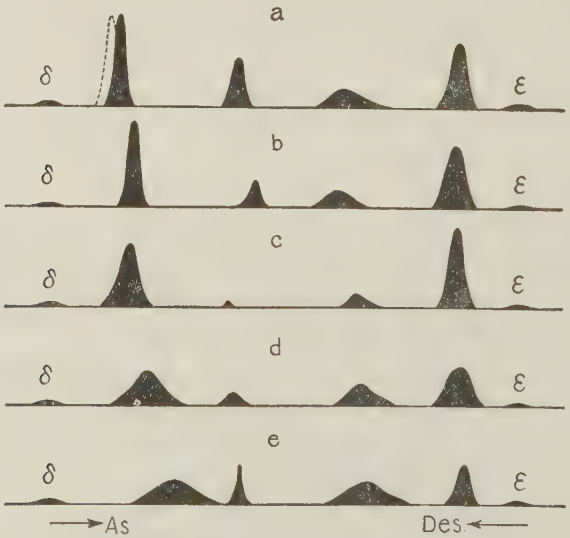


FIG. 1. Electrophoretic patterns of the mixture of β-lipovitellin (β-lv) and polyanions (in carbonate buffer, pH 9.8 and μ 0.1): a, β-lv (0.80 per cent)+RNA (1.35 per cent); b, β-lv (0.80 per cent)+heparin (0.33 per cent); c, β-lv (0.80 per cnt)+alginic acid (0.067 per cent); d, β-lv (0.80 per cent) +alginic acid (0.20 per cent); e, β-lv (0.80 per cent)+alginic acid (0.33 per cent). The peak represented by dotted line shows the position o. β-lv in the free β-lv experiment.

could be found in phosphate buffer of pH 7.8 and μ 0.25 as well as the case of heparin, but, in this case, no precipitate was formed during dialysis.

As the alginic acid concentration was increased, the following changes were observed; the increase in the mobility of slow ascending boundary, the decrease in the area of slow descending boundary, the increase in the area of fast descending boundary, and the increase in the area difference between fast ascending and fast descending boundaries. These variations occurred linearly with increase in the alginic acid concentration.

Throughout these experiments, only two boundaries, aside from the δ and ε effects, appeared in each side of the channel.

DISCUSSION

Reversible Formation of Soluble Complexes—Electrophoresis on the mixtures of β-lipovitellin and a series of polyanions showed that the presence of polyanion accelerated the β-lipovitellin peak in the ascending limb of the electrophoretic cell. The acceleration became progressively greater as the polyanion concentration was increased. In contrast to the changes observed in the ascending mobilities, there were no major changes evident in the corresponding descending mobilities of β-lipovitellin, with the exception of an appreciable increase at pH 7.8. These phenomena are explicable by considering the reversible formation of soluble complexes between components. Namely, according to Longsworth and MacInnes (*1*), the present system corresponds to the following interacting case; the complex is formed reversibly between β-lipovitellin (P) and polyanions (A), as

$$P + \nu A \rightleftarrows PA_\nu,$$

in which the velocity constants are both large for the forward and reverse reactions and of the same order of magnitude, hence the equilibrium is adjusted as rapidly as required by the electrophoretic separation of the components. Consequently, the complexes, which are in equilibrium with the free proteins and polyanions in the solution, may dissociate rapidly into its components, depending on the depletion of the free proteins or polyanions occurred in the concentration gradient.

Similar types of interaction were observed in the systems of ovalbumin and yeast nucleic acid (*1*), serum β-lipoprotein and heparin (*11*), and serum albumin and methyl orange (*13*). As explained in detail in these cases, electrophoresis of the interacting system of this kind results in the dissymmetry as observed here between the ascending and descending boundaries.

Influences of pH and Ionic Strength on Interaction—In the system of β-lipovitellin and heparin, a marked increase in mobility of β-lipovitellin, observed in the ascending limb at pH 9.8 and μ 0.1, was somewhat reduced by increasing the ionic strength from 0.1 to 0.3, whereas at pH 7.8, a marked increase was found even at an ionic strength of 0.3. A similar influence of pH and ionic strength on the interaction was also found in the system of β-lipovitellin and alginic acid. At pH 7.8, the mixture of β-lipovitellin and

heparin formed a precipitate which could be dissolved again by raising the pH. From these results, it is obvious that the present interaction is intensified by lowering the pH and weakened by increasing the ionic strength. Such an influence of pH and ionic strength found in the interaction suggests that the interaction is electrostatic nature. The similar behaviors of interaction were reported in the systems of serum albumin and DNA (*2*), RNA (*4*), and heparin (*6*).

However, in contrast with the case of serum albumin*, the present interaction does not disappear even at such considerable high pH** and ionic strength as 9.8 and 0.3, respectively. This is probably due to the contribution of van der Waals forces acting as another factor besides the electric forces, which are evidently expected in the present system consisted of a lipoprotein and polyanions such as heparin or alginic acid. Thus, it is suggested that the high affinity to polyanions, observed even at the considerably alkaline side of the isoelectric point, may be a characteristic nature common to lipoproteins. An evidence to this presumption is found in a recent work of Bernfeld *et al.* (*11*) that β-lipoglobulin is the only protein in human serum to interact with heparin in the range of pH 7.5 to 8.6.

Comparison of Affinities among Three Polyanions—An addition of 33 mg. alginic acid to 80 mg. of β-lipovitellin caused the increase of about 5 units in the ascending mobility of β-lipovitellin at pH 9.8 and μ 0.1. With heparin, the increase of about 2 units was observed at the same condition. On the other hand, no increase was found by an addition of 33 mg. RNA.

RNA, heparin, and alginic acid contain phosphoryl, sulfate, and carboxyl groups in their molecules, respectively***, all of which dissociate completely at pH 9.8. Thus, it is of interest that there is a marked difference among the increases in the ascending mobility of β-lipovitellin, depending on the difference in the charged groups or the residues of the polyanions.

Now, the mean equivalent weights per one charged group in the respective polyanion are able to be calculated from their molecular formula, as follows: RNA, 337****; heparin, 164; alginic acid, 176. Assuming that the formed complexes have a constant frictional coefficient, it is expected that the increments in the ascending mobility of β-lipovitellin brought about by a same quantity of heparin and alginic acid are nearly equal to each other, since both have an almost equal equivalent weight. But, heparin showed a considerably lower increment than alginic acid by an addition of same quantity with the latter. On the other hand RNA represented the

* In the case of serum albumin, the complexes were formed only below pH 5.5 and μ 0.2 (DNA) (*2*), pH 5.6 and μ 0.1 (RNA) (*4*), and pH 6.2 and μ 0.15 (heparin) (*6*), respectively.

** The isoelectric point of β-lipovitellin is pH 5.9 (*12*).

*** Heparin contains one carboxyl group besides two sulfate groups in a disaccharide unit (*14*).

**** A mean value of the four nucleotides.

increment less than one unit at the most even by an addition of 135 mg., although the mean equivalent weight of RNA is about twice the other two. Thus, defining the affinity of polyanions to β-lipovitellin as the combined number of mean equivalent unit, a following order is found in the affinities of the respective polyanion; alginic acid>heparin>RNA.

Non-Displacement of the Lipid Constituent of β-Lipovitellin by Polyanions—β-Lipovitellin contains about 40 per cent lipid, a great part of which is lecithin (*12*, *15*). If the treatment of β-lipovitellin with polyanions resulted in the displacement of the lipid constituent by polyanions, some new peak corresponding to free lecithin or lecithin-polyanion complex would appear in the electrophoretic pattern. However, only two peaks, aside from the δ and ε effects, were always observed throughout the experiments. It was moreover confirmed at pH 9.8 and μ 0.1 that there was no appearance of the peak corresponding to the free lecithin* or the lecithin-polyanion complex. Therefore, it can be concluded that β-lipovitellin formed the complex with polyanions without giving rise to any recognizable displacement of the lipid constituent by polyanions.

β-Lipovitellin used here is a material from which a great quantity of weakly combined lipid was removed by ether extraction (*12*). Therefore it is considered that the lipid fraction contained about 40 per cent in β-lipovitellin combines rather firmly to the protein carrier**. However, the egg lipoprotein prepared by chemical fractionation without ether extraction represented about 85 per cent lipid content***. And, also, it has been recently reported that the egg lipoprotein obtained by means of a preparative ultracentrifugation contains 89 per cent lipid (*16*). Accordingly, it is considered to be of interest to study the behavior of this weakly combined lipid against polyanions.

An Attempt of Quantitative Interpretation of Interaction—The changes in the electrophoretic patterns observed by varying the mixing ratio of interacting components provide some informations on the interaction, since the changes are closely connected with the complex formation in the system. However, in the present interacting system such as consisted of a protein and large polyanion, it is generally difficult to solve the problem except the special case, since the participations of some complicated factors are considered**** (*2*, *17*).

In a simple case that a single complex is in equilibrium with both free protein and polyanion, it becomes possible to know the composition of

* The free lecithin (extracted from egg yolk) represented −13.8 units of ascending and −12.7 units of descending mobilities at pH 9.8 and μ 0.1.

** Likewise, this lipid fraction could not be removed from the protein moiety by an addition of detergent such as sodium dodecyl sulfate (The author, unpublished data).

*** The author, unpublished data.

**** An application of the method of Smith and Briggs (*13*) to the present data was unsuccessful in obtaining the binding constant. In a interacting system containing large polyanion, it is considered that the number of combined polyanion is rather small and the electrostatic repulsions of neighboring groups must be important for a polyanion.

complex and the equilibrium constant in the interaction (*17*). The calculation was performed by using the data given in Table III*, in order to inspect whether the interacting system of β-lipovitellin (P) and alginic acid (A) is conformable to this case. However, it was impossible to explain the present data consistently by the existence of only one species of complex throughout the examined range of mixing ratio. The obtained result suggested that the complex of PA_2 type exists dominantly in the region of low polyanion concentration, while at high polyanion concentration, the PA_3 type becomes predominant.

SUMMARY

1. Electrophoreses on the mixtures of β-lipovitellin and a series of polyanions (RNA, heparin, and alginic acid) have represented the reversible formation of soluble complexes between the components.

2. The influences of pH and ionic strength to the interaction have shown that not only the electrostatic forces but also the van der Waals forces contribute to stabilize the interaction.

3. The affinities of three polyanions to β-lipovitellin have been compared by their mean equivalent weights.

4. From the electrophoretic patterns, it has been concluded that the polyanions form the complex with β-lipovitellin without splitting off the lipid-protein bond.

5. An attempt of quantitative interpretation of the interaction has been made.

The author wishes to express his sincere thanks to Prof. N. Ui of Gumma University for his valuable suggestions.

REFERENCES

(*1*) Longsworth, L. G., and MacInnes, D. A., *J. Gen. Physiol.*, **25**, 507 (1942)
(*2*) Goldwasser, E., and Putnam, F. W., *J. Phys. Colloid Chem.*, **54**, 79 (1950)
(*3*) Fleming, M., and Jordan, D. O., *Discussion Faraday Soc.*, **13**, 217 (1953)
(*4*) Nath, P., Cohly, M. A., and Giri, K. V., *J. Indian Inst. Sci.*, **37**, 163 (1955)
(*5*) Chargaff, E., Ziff, M., and Moore, D. H., *J. Biol. Chem.*, **139**, 383 (1941)
(*6*) Gorter, E., and Nanninga, L., *Discussion Faraday Soc.*, **13**, 205 (1953)
(*7*) Meyer, K., *Discussion Faraday Soc.*, **13**, 271 (1953)
(*8*) Klotz, I. M., in Neurath, H., and Bailey, K., The Proteins, Academic Press, New York, Vol. **I**, Part **B**, p. 727 (1953)
(*9*) Rosenberg, R. M., Lever, W. F., and Lyons, M. E., *J. Am. Chem. Soc.*, **77**, 6502 (1955)
(*10*) Oncley, J. L., Walton, K. W., and Cornwell, D. G., *J. Am. Chem. Soc.*, **79**, 4666 (1957)
(*11*) Bernfeld, P., Donahue, V. M., and Berkowitz, M. E., *J. Biol. Chem.*, **226**, 51 (1957)
(*12*) Sugano, H., *J. Biochem.*, **45**, 393, 667 (1958) **46**, 417 (1959)

* In the calculation, the molecular weight of β-lipovitellin was presumed as 300,000 from the sedimentation constant (*12*), and that of alginic acid was assumed as 15,000 (*18*).

(*13*) Smith, R. F., and Briggs, D. R., *J. Phys. Colloid Chem.*, **54**, 33 (1950)
(*14*) Jorpes, J. E., Bostrom, H., and Mutt, V., *J. Biol. Chem.*, **183**, 607 (1950)
(*15*) Fevold, H. L., and Lausten, A., *Arch. Biochem.*, **11**, 1 (1946)
(*16*) Nichols, A. V., Rubin, L., and Lindgren, F. T., *Proc. Soc. Exptl. Biol. Med.*, **85**, 352 (1954)
(*17*) Alberty, R. A., and Marvin, H. H., *J. Phys. Colloid Chem.*, **54**, 33 (1950)
(*18*) Endo, K., *J. Chem. Soc. (Japan)*, **72**, 968 (1951)

The Journal of Biochemistry, Vol. 46, No. 5, 1959

STUDIES ON AMINE OXIDASE: EFFECTS OF CHLORPROMAZINE AND ITS ANALOGUES

By HISASHI NAKAJIMA

(From the Department of Neuropsychiatry, Osaka University, Medical School, Osaka)

(Received for publication, July 11, 1958)

Serotonin, noradrenaline and histamine are thought to play an important role in the nervous system (*1*, *2*). The physiological role of amine oxidase, which catalyzes the oxidative deamination of such biologically active amines, has been the subject of controversy (*3*, *4*), and various inhibitors have been used for the study of this enzyme. In previous studies (*5*, *6*) it was shown that chlorpromazine inhibits the oxidative deamination of serotonin both *in vivo* and *in vitro*. Many papers have recently appeared with respect to the biochemical actions of this new psychotherapeutic drug (*7*) and evidence has been accumulated showing that it strongly inhibits various enzymes and enzyme systems. However the mechanism of this inhibition is still unknown. It seemed therefore to be of interest to know the mechanism of inhibition of amine oxidase by chlorpromazine.

This paper presents the results of the inhibitory effect of chlorpromazine on monoamine oxidase and of the effect of sulfhydryl compounds and FAD on the inhibition of the enzyme by chlorpromazine *in vitro*. Studies have also been made on the effect on monoamine oxidase of various compounds structually related to chrorpromazine and on the effect of chlorpromazine on monoamine oxidase *in vivo*.

METHODS

*Preparation of Monoamine Oxidase (MO)**—The following methods were used:

Partially purified enzyme was prepared from hog liver according to Kohn's method (*8*). *Mitochondrial soluble MO* was prepared from guinea pig liver by the method of Kobayashi and Schayer (*9*) but the dialysis time was shortened. *Soluble MO from "high speed supernatant fluid"* of guinea pig liver homogenate was obtained by the method of Weissbach *et al.* (*10*).

Brain mitochondrial fraction was prepared from guinea pig according to the procedure of Brody and Bain (*11*).

Assay of the Enzyme Activity—1. *Oxygen Uptake*: This was measured in a Warburg manometer at 37° with air as the gas phase. The reaction mixture contained in the main chamber: 1.0 ml. of enzyme preparation, 0.2 ml. of 10^{-2} *M* KCN, 0.2 ml. of 0.5 *M*

* The abbreviation used in this communication are MO and DO, monoamine and diamine oxidase, respectively; FAD, flavine adenine dinucleotide; 5-HIAA, 5-hydroxyindoleacetic acid; and 5-HIAAl, 5-Hydroxyindole acetaldehyde.

phosphate buffer of pH 7.0 and 0.2 ml. of an aqueous solution o the drug under investigation. The center well contained 0.1 ml. of 50 per cent KOH. The side arm contained 0.3 ml. of amine solution (3 μM). The final volume was adjusted to 2.1 ml. with water. Serotonin creatinine sulfate, tyramine hydrochloride or noradrenaline bitartrate, were used as substrates.

2. *Disappearance of Serotonin and Formation of 5-Hydroxyindole Acetaldehyde (5-HIAAl)*; The reaction mixture contained 1.0 ml. of enzyme, 1.0 ml. of serotonin (2 μM), 0.3 ml. of chlorpromazine, 0.3 ml. of 0.5 *M* phosphate buffer of pH 6.9, and 0.7 ml. of semicarbazide (5 mg.), and the final volume was adjusted to 3.0 ml. with water. At the end of the reaction period, the mixture was deproteinized by adding 0.6 ml. of 10 per cent zinc sulfate and brought to pH 10 with 1.0 ml. of saturated sodium carbonate solution. The chlorpromazine was removed by shaking with 15 ml. of chloroform. Interference of chlorpromazine with colour development was completely prevented by this procedure. The aqueous phase was centrifuged and 2.5 ml. of the supernatant fluid was used for determination of serotonin and 1.0 ml. for determination of 5-HIAAl. Serotonin was determined by the modified Udenfriend's method (*12*) and 5-HIAAl by the method of Weissbach *et al.* (*10*).

RESULTS

Effect of Chlorpromazine on Monoamine Oxidase in Vitro—In a partially purified MO preparation from the hog liver, when serotonin was used as substrate, oxygen consumption was decreased by about 50 per cent in the presence of 10^{-3} *M* chlorpromazine (Fig. 1). This degree of inhibition was in

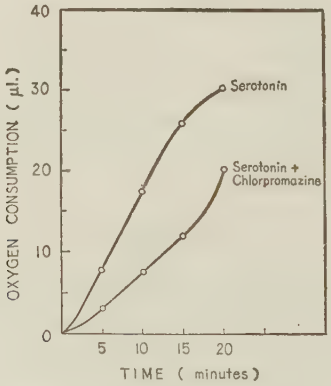


FIG. 1. Inhibition of hog liver MO by chlorpromazine. The reaction vessels contained 0.1 ml. of the partially purified enzyme from hog liver, 3 μM serotonin and 10^{-3} *M* of chlorpromazine, pH 7.0, 37°. In the control chlorpromazine was replaced by water.

accordance with the data obtained by the colorimetric determination of 5-HIAAl formed and of serotonin destroyed (Table I). Chlorpromazine inhibited the oxidation by both guinea pig liver and brain mitochondrial MO of serotonin, tyramine and noradrenaline (Table II).

TABLE I

Inhibition of the Guinea Pig Liver MO by Various Concentration of Chlorpromazine

Concentration of chlorpromazine	5-HIAAl formation (Optical density difference)	Serotonin destruction ($\mu g.$)
—	.057	278
10^{-3} *M*	.011	167
5×10^{-4} *M*	.016	241
10^{-4} *M*	.042	241
10^{-5} *M*	.042	235

TABLE II

Effect of Chlorpromazine on Guinea Pig Liver and Brain Mitochondrial MO

	Liver		Brain	
	O_2 uptake ($\mu l./hr.$)	Inhibition (%)	O_2 uptake ($\mu l./hr.$)	Inhibiton (%)
3 μM serotonin	51.6		29.0	
3 μM serotonin +1 mM chlorpromazine	30.9	44.0	14.5	50.0
3 μM tyramine	96.9		43.0	
3 μM tyramine +1 mM chlorpromazine	57.6	43.5	14.7	66.0
3 μM noradrenaline	38.4		28.5	
3 μM noradrenaline +1 mM chlorpromazine	20.1	47.5	5.0	82.0

The reaction vessels contained 1.0 ml. of soluble MO from "high speed supernatant fluid" of guinea pig liver homogenate or brain mitochondrial fraction.

In the next experiment, the possibility that the inhibition of MO might be reversed by cysteine or glutathione was studied, since MO had been known to be sensitive to SH-reagents (*13*). However, 2×10^{-5} *M* of glutathione had no effect in preventing the inhibition. The influence of cysteine could not be demonstrated, because cysteine itself showed an inhibitory effect on MO and the oxidation of cysteine by hog liver homogenate was also depressed by chlorpromazine. FAD, which is supposed to be a cofactor of MO (*14*, *15*), had no effect on the inhibition of MO by chlorpromazine.

To analyze the effect of substrate concentration on the reaction velocity, oxygen consumption was measured during a twenty minutes incubation period using various concentrations of serotonin. When the reaction velocity, v, was plotted against concentration of substrate, $[S]$, in a "double reciprocal" manner, results fell on a parabolic curve. When $1/[S]^2$ was plotted in place of $[1/S]$, the data fell on a straight line, as shown in Fig. 2. The reason for this intriguing relationship remains for investigations in future.

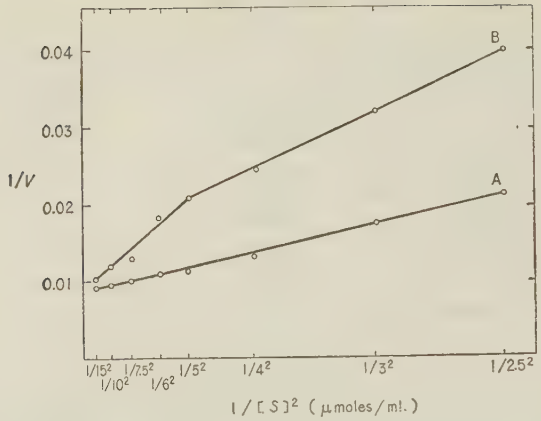


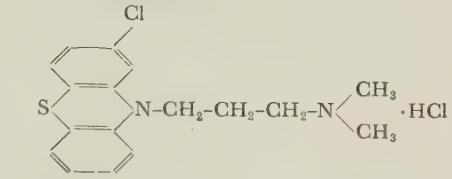
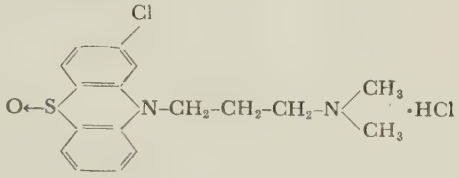
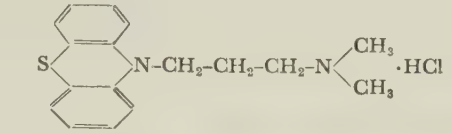
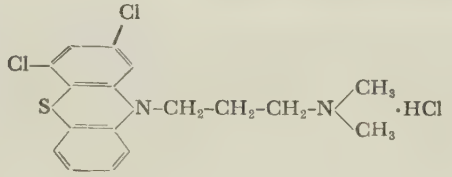
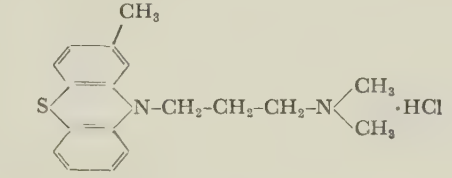
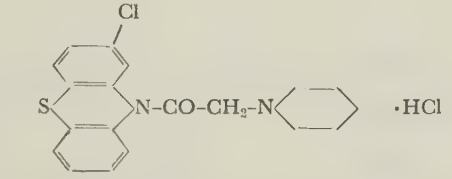
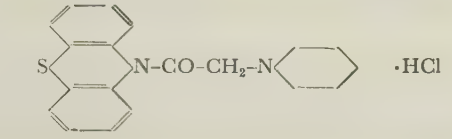
FIG. 2. Plot of reciprocal of reaction velocity (V) against square of reciprocal of substrate concentration ($[S]$) for hog liver MO with and without 10^{-3} M of chlorpromazine. A, control, no chlorpromazine; B, chloropromazine added; Substrate, serotonin. Reaction velocity was calculated from the oxygen consumption during a 20 minutes' incubation period.

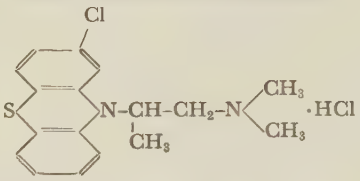
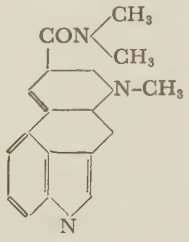
Inhibitory Effect of Various Phenothiazine Derivatives—In an attempt to compare the effect of chlorpromazine and of its analogues on MO, phenothiazine derivatives structurally related to chlorpromazine were added to the reaction mixture. Results are shown in Table III. Per cent inhibition was calculated from the oxygen uptake during a 30 minutes period incubation in the presence of 3 μM serotonin. Similar results were obtained from determination of 5-HIAA formation. Chlorpromazine was the most effective inhibitor of the compounds studied. Chlorpromazine sulfoxide, in which the S atom of phenothiazine nucleus is oxidized, produced negligible inhibition. Promazine, which lacks the Cl in the 3-position of chlorpromazine, was not so effective as chlorpromazine. Substitution of a methyl group in the 3-position of the phenothiazine nucleus resulted in an increase in inhibition; methylpromazine was comparable in effectiveness to chlorpromazine. The presence of Cl in the 1- and 3- positions of the nucleus (as in dichlorpromazine) decreased the per cent inhibition. When the dimethylaminopropyl in the side chain was replaced by a piperidinoacetyl group (compounds 6 and 7), inhibition was diminished. These relationships will be discussed later.

Since reserpine and frenquel have been widely used as psychotherapeutic drugs and lysergic acid diethylamide (LSD) has been studied as a hallucinogenic agent, their effects on serotonin oxidation were also investigated. Reserpine and frenquel showed no effect on the degradation of serotonin. In the presence of 50 μg. of LSD there was a stimulation by about 14 per cent in serotonin breakdown. However if liver homogenate instead of purified enzyme was used, LSD was slightly inhibitory. There is as yet no explanation for this contradictory result.

Table III

The Inhibitory Effects of Various Phenothiazine Derivatives on Monoamine Oxidase

Compound	Structure	Per cent inhibition
1. Chlorpromazine		50
2. Chlorpromazine sulfoxide		8
3. Promazine		23
4. Dichlorpromazine		16
5. Methylpromazine		34
6. 3-Chloro, 10-(piperidinoacetyl) phenothiazine		15
7. 10-(Piperidinoacetyl) phenothiazine		19

Compound	Structure	Per cent inhibition
8. Phenergan		32
9. LSD*		−14

The reaction vessels contained 1 ml. of partially purified hog liver MO, 0.2 ml. of 10^{-2} *M* KCN, 0.2 ml. of 0.5 *M* phosphate buffer, pH 7.0, 0.2 ml. of an aqueous solution of the drug (10^{-3} *M*), and 0.3 ml. of serotonin (3 μM); final volume, 2.1 ml. Per cent inhibition was calculated from oxygen uptake during a 30 minutes incubation period.

* Amount of LSD added was 50 μg.

In Vivo Experiments—Since administration of chlorpromazine decreased the excretion of 5-hydroxyindoleacetic acid in the urine of the animals which had been injected with serotonin (*6*), effect of chlorpromazine on MO *in vivo* was investigated. Guinea pigs weighing 180–200 g. were injected intraperitoneally with 20 mg./kg. body weight of chlorpromazine and sacrificed one hour after the injection. The brain and liver were removed rapidly and homogenized with water. Enzyme activity was assayed by following the rate of serotonin destruction and of 5-HIIAl formation. No significant difference was observed between the injected and control groups in serotonin destruction by the homogenates of brain and liver as shown in the Fig. 3. There was however slight decrease in 5-HIAAl formed from serotonin by the brain homogenate of injected animals. ($P<0.01$), though decrease was not observed in the liver homogenates (Fig. 4).

When a homogenate was used as the enzyme preparation, disappearance of serotonin does not appear to correspond directly to the activity of MO. This is shown by Udenfriend's finding that 5-HIIA accounts for only 30 per cent of the metabolized serotonin (*16*) and from our experiments (described below) with tissue homogenates using high-voltage paper electrophoresis.

Rats of about 200 g. were killed and the brain, liver, kidney, intestine, adrenal glands and heart immediately removed. The homogenates of these organs were incubated with 2 μM of serotonin for 150 minutes at 37°. and

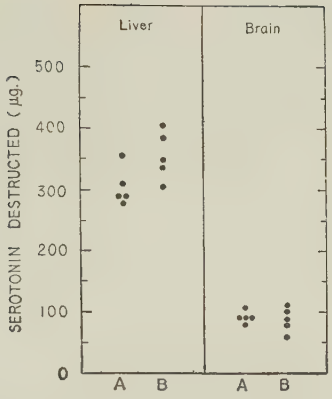


FIG. 3. Destruction of serotonin by liver and brain homogenates from guinea pig injected with chlorpromazine.

The brain and liver of guinea pig killed one hour after injection of chlorpromazine (20 mg./kg. body weight) were removed rapidly. They were homogenized with 2 volumes (brain) or 99 volumes (liver) of distilled water. To one ml. of the homogenate, 1 μM (brain) or 2 μM (liver) of serotonin and 50 mg. of semicarbazid were added and incubated for 1 hour (brain) or 45 minutes (liver) at 37°, pH 6.9. Serotonin was measured by U d e n f r i e n d's method (*12*). A, control group; B, injected group.

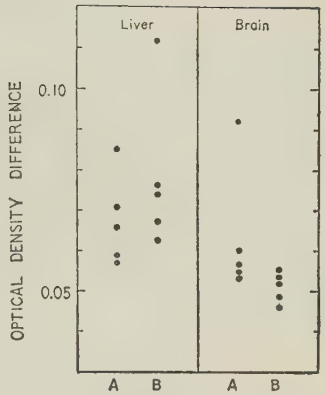


FIG. 4. Formation of 5-HIIAl from serotonin by the liver and brain homogenates from guinea pig after injection with chlorpromazine.

Condition as in Fig. 3. Optical density of 5-HIIAl semicarbazone was read at 440 mμ in a Beckman spectrophotometer.

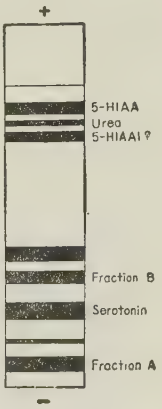


FIG. 5. Diagram of high voltage electrophoresis paper chromatogram of serotonin metabolites (4,000–5,000 v./40 cm., 30 minutes, pH 3.9).

the reaction mixtures deproteinized with 5 volumes of acetone. The dried filtrates were subjected to high-voltage electrophoresis (4,000—5,000 v./40 cm., 30 minutes) by the method of H e i l m e y e r *et al.* (*17*). Several fractions

besides serotonin and 5-HIAA were detected with Ehrlich's *p*-dimethylaminobenzaldehyde reagent. These fractions included two coloured fractions, Fractions A and B, as shown in Fig. 5. Fraction A was found only in materials from intestine and adrenal gland but B was present in materials from all organs tested. Both fractions gave positive reactions with the following tests: *p*-dimethylaminobenzaldehyde reaction, *α*-nitroso-*β*-naphthol-HNO_2 reaction, ninhydrin reaction, reaction with diazotized sulfanillic acid (acidic), Dragendorff's reaction and Simmon's reaction (Table IV). Moreover the formation of these substances from serotonin were not inhibited by iproniazid.

TABLE IV

Color Reaction of Fraction A and B with Various Reagents

Reagents	Color of Fraction A and B
p-Dimethylaminobenzaldehyde	Blue violet
α-Nitroso-*β*-naphthol-HNO_2	Violet
Diazotized sulfanilic acid (acidic)	Orange
Ninhydrin	Yellowish brown
Dragendorff	Orange
Simmon	Violet

TABLE V

Paper Chromatographic Behavior of Fraction A and B

Substance	Isopropanol : ammonia : water (20 : 1 : 2) (R_f)	Butanol saturated with 14% ammonia (R_f)	Butanol saturated with 1 *N* HCl (R_f)
Bufotenin	0.91	0.89	0.20
Serotonin	0.62	0.64	0.17
Fraction A	0.45	0.49	0.06
Fraction B	0.63	0.68	0.19
N-Methylserotonin*	0.55	0.75	0.19

* R_f value of *N*-methylserotonin is cited from data of Bampus and Page (*18*), since the synthetic substance could not obtained.

It was then presumed that either Fraction A or B may be *N*-methylserotonin, excretion of which in human urine was reported by Bampus and Page (*18*). Part of each fraction was eluted from the paper and rechromatographied on paper. R_f values of these substances are shown in Table V. Fraction B appeared to correspond to 5-hydroxy-*N*-methylindolethylamine (*N*-methylserotonin). Fraction A has yet been identified.

DISCUSSION

Degradation of serotonin by MO was inhibited by chlorpromazine as shown above. According to I c h i m a r u* chlorpromazine also inhibits acetylcholinesterase from human brain by about 50 per cent at concentration of 2.5×10^{-4} *M* and chlorpromazine sulfoxide is as effective on AchE though chlorpromazine-*S*, *N*-dioxide is not. The inhibitions of both MO and AchE are interesting in view of the fact that these enzyme play an important role in the central nervous system, and that there are reports of abnormally high activity of MO in the blood (*19*) and liver (*20*) in a high percentage of schizophrenics, although it is uncertain whether the therapeutic effects of drugs are attributable to inhibition of the enzymes.

It was reported that the inhibitory effect of chlorpromazine on succinic dehydrogenase was reversed by cysteine and glutathione (*21*) and that D-amino acid oxidase were inhibited by chlorpromazine which competes with FAD (*22*). However, these facts could not be shown with MO.

Iproniazid, one of the most potent inhibitors of MO, has been reported to be benificial to depressive patients (*23*), and phenylmethylpropamine is known as a stimulant of the central nervous system. It is an apparent discrepancy that chlorpromazine, and iproniazid and phenyl-methylpropamine inhibit MO. However, the two latters inhibit MO competitively and chlorpromazine probably reacts with the enzyme at the site other than active centre in a different manner to iproniazid (*24*). Moreover, serotonin is probably attacked by several enzymes other than MO, so it is unreasonable to attempt to explain the effects of these drugs on serotonin metabolism only by their actions on MO. In this connection it is noteworthy that several unidentified metabolites of serotonin were detected *in vitro*.

From the data on the effects of chlorpromazine analogues on MO, the significance of following structual moieties comes into discussion; 1) the S atom in the phenothiazine nucleus, 2) substitutions in the 3-position of the phenothiazine nucleus, and 3) the structure of the side chain. Firstly, if the S atom is coordinated with the O atom, the inhibitory activity was decreased. Therefore it is likely that the lone pair of electrons around S may participate in the reaction of the drug with the enzyme. Secondly, from the observation that compounds with a Cl or CH_3-group in position 3 were effective than promazine, it might be suggested that a shift of electrons from S occurred by such a substitution that this caused increased inhibitory activity. Further comparative studies on the effect of compounds with different halogens in the 3-position should, however, be made. The weak inhibitory effect of dichlorpromazine is probably due to a a steric hindrance of Cl in position 1. Thirdly, with regard to the side chain, the dimethylaminopropyl group may have an affinity for the enzyme protein. The fact that methylene blue also strongly inhibits MO *in vitro* (*25*) may support the assumption that the phenothiazine nucleus plays a more

* Personal communication.

important role in inhibition than does the side chain.

Bernsohn (*26*) and Abood (*27*) recently investigated inhibition of cytochrome oxidase, ATPase and oxidative phosphorylation in relation with the chemical structure of phenothiazine derivatives. They showed that chlorpromazine, phenergan, promazine and chlorpromazine sulfoxide are inhibitory, decreasing in that order. This finding agrees with the results presented in this paper. In another experiments we carried out similar studies with respect to diamine oxidase (DO), and a similar tendency was recognized in the correlation of structure of phenothiazine derivatives to inhibition of DO as found in the case of MO, although DO was inhibited somewhat less effectively (Table VI).

TABLE VI

The Inhibitory Effects of Various Phenothiazine Derivatives on Diamine Oxidase

Compound	Per cent inhibition
1. Chlorpromazine	36
2. Chlorpromazine sulfoxide	5
3. Promazine	−14
4. Dichlorpromazine	14
5. Methylpromazine	35
6. 3-Chloro, 10-(piperidinoacetyl) phenothiazine	8
7. 10-(Piperidinoacetyl) phenothiazine	9.5
8. 3-Chloro, 10-dimethylaminoacetyl phenothiazine	32
9. Phenergan	20.5
10. Reserpin	4
11. LSD*	11

The reaction vessels contained 1 ml. of hog kidney DO, 0.2 ml. of 0.5 *M* phosphate buffer, pH 7.0, 0.2 ml. of an aqueous solution of the drug (10^{-3} *M*), and 0.3 ml. of histamine (3 μM); final volume 2.1 ml.. Per cent inhibition was calculated from oxygen uptake during a 40 minutes incubation period.

* Amount of LSD added was 50 μg.

Humans and animals after administration of chlorpromazine excrete decreased amounts of 5-HIAA in the urine as shown by previous results from this laboratory. This could not be confirmed by *in vivo* experiments on MO inhibition, though a slight decrease in 5-HIAAl formation by the brain homogenates of guinea pig treated with chlorpromazine was obserbed. From the experiment with tissue homogenates using a high voltage paper electrophoresis apparatus, it was found that several metabolites other than 5-HIAA were produced from serotonin. It is assumed that serotonin has several metabolic pathways through 5-HIAAl, 5-HIAA, methylserotonin *etc.* in various organs. Therefore, disappearance of serotonin, is not to correspond

with activity of MO, when a homogenate was used as an enzyme preparation. This may explain the inconsistent observation that in brain homogenates from guinea pig after injection with chlorpromazine the formation of 5-HIAAl was decreased while serotonin destruction was not.

Recently Costa and Rinaldi (*28*) reported that, in various brain parts of animals pretreated with chlorpromazine, the administration of 5-hydroxytryptophan, precursor of serotonin, caused increase of serotonin concentrations as compared with control animals which were treated only with 5-hydroxytryptophan. Their result may also be due to an inhibitory effect of chlorpromazine on MO *in vivo*.

SUMMARY

1. Monoamine oxidase from the liver and brain was inhibited by chlorpromazine. This inhibition was not reversed by cysteine, glutathione or FAD.

2. Monoamine oxidase was inhibited in decreasing order by chlorpromazine, phenergan, promazine, dichlorpromazine, 3-chloro, 10-piperidinoacetyl phenothiazine and chlorpromazine sulfoxide. Lysergic acid diethylamide stimulated MO slightly.

3. Chlorpromazine had no effect liver MO *in vivo*, while it slightly decreased 5-hydroxyindole acetaldehyde formation from serotonin by brain MO.

4. Several metabolites of serotonin were found in vitro in homogenates of various organs. One of these seemed to be *N*-methylserotonin.

REFERENCES

(*1*) Sano, I., and Kakimoto, Y., *Saishin Igaku*, **11**, 1483 (1956)
(*2*) Euler, von U. S., Noradrenaline, Charles C Thomas Publisher, Springfield (1956)
(*3*) Furchgott, R. F., *Pharmacol. Rev.*, **7**, 183 (1955)
(*4*) Satake, K., *Yakugaku no Shimpo*, **4** (**I**), 39 (1955)
(*5*) Sano, I., Kakimoto, Y., Okamoto, T., Nakajima, H., and Kudo, Y., *Schweiz. Med. Wschr.*, **87**, 214 (1957)
(*6*) Sano, I., Kakimoto, Y., and Okamoto, T., *Der Nervenarzt*, **28**, 363 (1957)
(*7*) Kakimoto, Y., and Nakajima, H., *Japan. J. Pharm. Chem.*, **29**, 346 (1957)
(*8*) Kohn, H. I., *Biochem. J.*, **31**, 1693 (1937)
(*9*) Kobayashi, Y., and Schayer R. W., *Arch. Biochem. Biophys.*, **58**, 181 (1955)
(*10*) Weissbach, H., Redfield, B. G., and Udenfriend, S., *J. Biol. Chem.*, **229**, 953 (1957)
(*11*) Brody, J. M., and Bain, J. A., *J. Biol. Chem.*, **195**, 685 (1952)
(*12*) Udenfriend, S., Weissbach, H., and Clark, C. T., *J. Biol. Chem.*, **215**, 337 (1955)
(*13*) Singer, T. P., and Barron, E. S. G., *J. Biol. Chem.*, **157**, 241 (1945)
(*14*) Hawking, J., *Biochem. J.*, **50**, 399 (1952)
(*15*) Koyanagi, T., and Ota, M., *Vitamines (Japan)*, **7**, 992 (1954)
(*16*) Udenfriend, S., and Titus, E., A Symposium on Amino Acid Metabolism, edited by W. D. McElroy and H. B. Bentley, The Johns Hopkins Press, Baltimore, 945 (1955)
(*17*) Heilmeyer, L., Clotter, R., Sano, I., Sturum, A., and Lipp, A., *Klin. Wschr.*, **32**, 831 (1954)

(*18*) Bampus, F. M., and Page, I. H., *J. Biol. Chem* **212**, 111 (1955)
(*19*) Buscaino, G. A., *Acta Neurol.* (*Napori*), **7**, 395 (1952)
(*20*) Takahashi, Y., *Folia Psychiat. Neurol. Japon.*, **10**, 263 (1956)
(*21*) Kurokawa, M., Naruse, H., Kato, M., Yabe, T., and Haba, R., *Psychiat. Neurol. Japan*, **59**, 20 (1757)
(*22*) Yagi, K., Nagatsu, T., and Ozawa, T., *Nature*, **177**, 891 (1956)
(*23*) Ferreira, A. J. D., and Freeman, H., *Am. J. Psychiat.*, **114**, 933 (1958)
(*24*) Davidson, A. N., *Biochem. J.*, **67**, 316 (1957)
(*25*) Philpot, F. G., *Biochem. J.*, **31**, 856 (1937)
(*26*) Bernsohn, J., Namajuska, I., and Cochrance, L. S. G., *Proc. Soc. Exptl. Biol. Med.*, **92**, 20 (1956)
(*27*) Abood, L. G., and Romanchek, L., *Ann. N. Y. Acad. Sci.*, **63**, 812 (1957)
(*28*) Costa, E., and Rinaldi, F., *Am. J. Physiol.*, **194**, 214 (1958)

The Journal of Biochemistry, Vol. 46, No. 5, 1959

ION EXCHANGE SEPARATION OF FRUCTOSE-1-PHOSPHATE USING BORATE AS ELUANT

By YOSHITO HARA

(From the Department of Biochemistry, Okayama University Medical School, Okayama)

(Received for publication, August 30, 1958)

Ion exchange separation of sugar phosphates by using dilute borate elution as eluant has been established by Khym *et al.* (*1–3*). Separation of fructose-1-phosphate (F-1-P), however, has not been reported in their papers. As the eluting agents, 1×10^{-3} *M* $K_2B_4O_7$ was used for the elution of glucose-6-phosphate (G-6-P) and 1×10^{-5} *M* $K_2B_4O_7$ for that of fructose-6-phosphate (F-6-P), but 1×10^{-4} *M* $K_2B_4O_7$ has not been used in their procedure. This communication describes that 1×10^{-4} *M* borate elution gave a good separation of F-1-P from other sugar phosphates.

EXPERIMENTALS

Glucose-1-phosphate (G-1-P), G-6-P, F-6-P and fructose-1,6-diphosphate (F-1, 6-diP) used as test materials were commercial products. F-1-P was prepared from F-1, 6-diP by using rabbit bone phosphatase (*4–6*). The sugar phosphate salts mixture (each containing 1 to 2 mg. of free sugar) were converted to the free acids by treating with the acid form of Dowex 50 and neutralized with dilute aqueous ammonia (pH 8.5).

A 200- to 400-mesh Dowex 1 resin was freed of fine particles by decantation of a stirred suspension, and slurried into a column 0.9×10.5 cm. The resin was converted to the chloride form by washing with 1 *N* HCl and washed thoroughly with water to remove excess hydrochloric acid.

The solution of test materials in dilute ammonia was passed through the column. Free sugars were removed with 100 ml. of 1×10^{-3} *M* ammonia. A succession of eluting agents, in its order described (Fig. 1), was carried on through the column to elute selectively the components of the mixture. One thousand ml. of each eluting agent was used and each effluent fraction, 50 ml. in volume in most of the experiments, was collected at a maximum rate of 1.5 ml. per minute.

Assay of the effluent samples, was conducted using the phenol-H_2SO_4 method (*7*) for aldose phosphates and the cysteine-carbazol-H_2SO_4 method (*8*) for ketose phosphates, optical densities being measured with a Beckman spectrophotometer. Wave lengths 490 and 560 mμ were used for aldose and ketose, respectively.

Each fraction obtained by the procedure shown in Fig. 1 was concentrated in vacuum and isolated as barium salt. After removal of barium with Dowex 50, the sugar phosphates were identified by paper chromatography. Modified methods of Bandurski *et al.* (*9*) and Burrows *et al.* (*10*) were used for this purpose. For the color development on paper, Burrows' method was used. Figs. 2 and 3 show the paper chromatograms of sugar phosphates. Since the R_f value of each sugar phosphate was found to be different

from that reported in original papers (*9, 10*), authentic samples were used for comparison.

RESULTS AND DISCUSSION

As shown in Fig. 1, F-1-P appeared only in $1\times10^{-4}\,M$ borate effluent

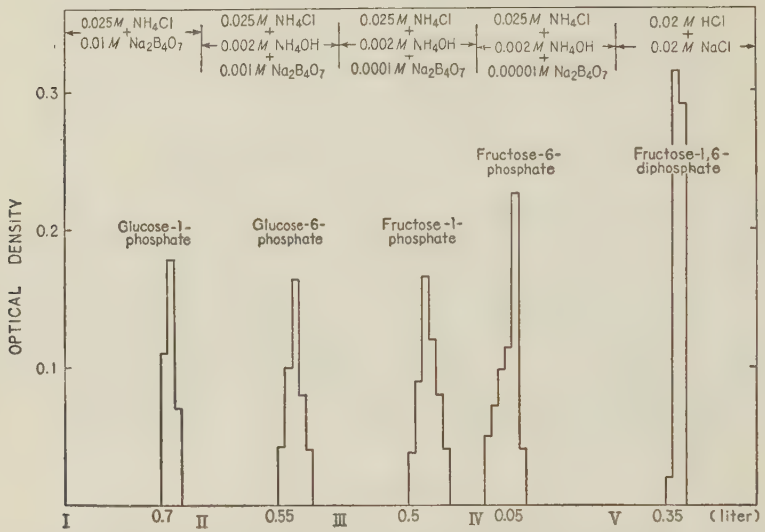


FIG. 1. Ion exchange separation of sugar phosphates. Exchanger, Dowex 1 (chloride), 200 to 400 mesh, 0.9×10.5 cm., flow rate 1 ml. per minute. The sample of each sugar phosphate contained about 1 to 2 mg. free sugar.

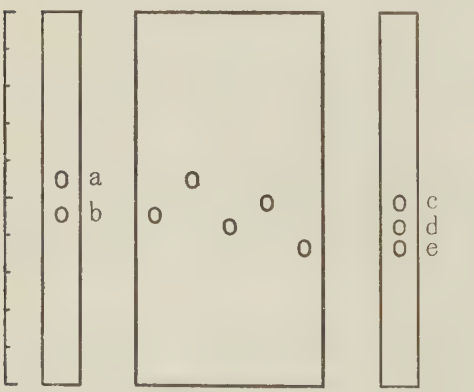


FIG. 2. One dimensional chromatogram (by Bandurski and Axelrod).
I, II, III, IV, V: Numbers of fractions mentioned in Fig. 1.

FIG. 3. One demensional chromatogram (by Burrows *et al.*).
III, IV: Numbers of fractions mentioned in Fig. 1.

and detected and identified by paper chromatography (Figs. 2 and 3). When the flow rate was kept at 3 to 3.5 ml. per minute, instead of 1.5 ml. per minute, there occurred an overlapping the peaks of F-6-P and F-1-P. Sodium borate buffer instead of potassium salt could be used without any significant differences. The recovery of sugar phosphates was 90 to 95 per cent.

Recently, Bartlett (*11*) has reported the separation of glycolytic intermediates including F-1-P by eluting with 0 to 1 *N* formic acid from Dowex-1 formate column. Because of the brevity of Bartlett's description, however, we cannot assess the merit of his method in comparison with the one described in the present paper.

SUMMARY

A method of ion exchange separation of sugar phosphates including F-1-P was described. The sugar phosphate in each fraction was identified by paper chromatography. The recovery of each sugar phosphates was 90 to 95 per cent.

REFERENCES

(*1*) Khym, J. X., and Cohn, W. E., *J. Am. Chem. Soc.*, **75**, 1153 (1953)
(*2*) Khym, J. X., Doherty, D. E., and Cohn, W. E., *J. Am. Chem. Soc.*, **76**, 5523 (1954)
(*3*) Colowick, S. P., and Kaplan, N. O., Methods in Enzymology, Academic Press, N. Y., Vol. **III**, p. 110 (1957)
(*4*) Neuberg, C., and Lustig, H., *J. Am. Chem. Soc.*, **64**, 2722 (1942)
(*5*) Martland, M. and Robison, R., *Biochem. J.* **23**, 237 (1929)
(*6*) Tanko, B., and Robison, R., *Biochem. J*, **29**, 961 (1935)
(*7*) Disch, Z., and Borenfreund, E., *J. Biol. Chem.*, **192**, 583 (1951)
(*8*) Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F., *Anal. Chem.*, **28**, 350 (1956); *Nature*, **168**, 167 (1951)
(*9*) Bandurski, R. S., and Axelrod, B., *J. Biol. Chem.*, **193**, 405 (1951)
(*10*) Burrows, S., Grylls, F. S. M., and Harrison, J. S., *Nature*, **170**, 800 (1952)
(*11*) Bartlett, G. R., *Division of Biol. Chem. of A. C. S.*, 39C (1957)

The Journal of Biochemistry, Vol. 46, No. 5, 1959

PURIFICATION OF LIPASE PRODUCED BY RHIZOPUS*

By SUEO TATSUOKA, AKIRA MIYAKE, SHYOZO WADA and ISUKE IMADA

(*From the Research Laboratories, Takeda Pharmaceutical Industries Ltd., Osaka*)

CHIKASHI MATSUMURA

(*From the Institute for Fermentation, Osaka*)

(Received for publication, September 3, 1958)

Previous studies of lipase were conducted only on the enzymes produced from animal organs and plant seeds. Of late, however, the enzymes produced by microorganisms (*1–3*) also have come to be studied.

Most recently, Matsumura (*4*) found that R-302, a strain belonging to *Rhizopus*, produced lipase of high activity and reported on the culture method and properties of the preparation.

The present authors produced lipase by tank culture of this *Rhizopus* and purified the product by fractionation with ammonium sulfate and subsequent adsorption by calcium phosphate, obtaining a preparation of several hundreds as high activity as the crude product has. This paper deals with the method and result.

EXPERIMENTS AND RESULTS

Assay—One ml. of an enzyme solution (pH 5.2) was shaken with 3 ml. of 0.2 *M* phosphate buffer, 7 ml. of water and 2 ml. of olive oil in an L-shaped shaking tube closed with a rubber stopper at 30° for one hour. The reaction mixture was poured out, the tube was rinsed with 20 ml. each of ethanol and ether, and the combined reaction mixture and rinsings were titrated with 0.1 *N* alcoholic potassium hydroxide. The activity of the enzyme was expressed in terms of unit: One ml. of the difference between the volume of the alkali solution consumed in the titration and the volume of the alkali solution used for titration of the blank solution was defined as one unit. Incidentally, the blank solution was produced by heating the enzyme solution before the reaction in order to destroy the enzyme therein.

Culture—R-302, a strain belonging to *Rhizopus*, was cultivated at 27–28° for 66–72 hours with aeration and agitation in 70 liter of a medium (pH 6.8) consisting of 9 per cent of cornsteep liquor, 3 per cent of ammonium sulfate, 12 per cent of dextrin, and 4.5 per cent of calcium carbonate.

* This work was presented at Symposium on the Medical Enzymology in Tokyo, in October, 1957.

Purification—a) Partial Purification of Lipase: The culture was filtered with 300 g. of Dicalite, 35 kg. of ammonium sulfate was dissolved in the filtrate, the resulting precipitate was filtered with Dicalite and the fiiltered cake was dissolved in 7 liter of water. The Dicalite suspended in the solution was removed by filtration and 3.5 kg. of ammonium sulfate was dissolved in the clear filtrate. The deposited precipitate was collected by centrifugation and extracted with 7 liter of ammonium sulfate solution containing 0.45 its volume of saturated ammonium sulfate solution in order to remove colored impurities. Ammonium sulfate was added to the extract, the resulting precipitate was collected and dissolved in a little amount of water, and the solution was dialyzed overnight in a bladder bag against 0.5 per cent solution of ammonium sulfate at 2-3°. The lipase solution thus obtained was diluted with distilled water to make the concentration of ammonium sulfate less than 0.3 per cent. A portion of 1,500 g. of calcium phosphate gel or 750 g. of calcium phosphate was added to the solution to adsorb all the

TABLE I

Purification of Rhizopus Lipase

Stage of purification		Total activity*	Yield (*per cent*)	Specific activity* (*unit/hr./mg.protein*)
Filtered broth		14,000,000		6
Precipitated with $(NH_4)_2SO_4$ and extracted with 45% sat. $(NH_4)_2SO_4$ solution		10,500,000	75	100
Adsorbed on $Ca_3(PO_4)_2$ and eluted with 1.0 *M* $(NH_4)_2SO_4$ solution		10,000,000	72	600
Fractionated with sat. $(NH_4)_2SO_4$ solution (45–70%)		9,000,000	64	1000
Refractionated with sat. $(NH_4)_2SO_4$ solution (60–65%)		2,000,000	14	2000
Chromatographed over $Ca_3(PO_4)_2$	α-lipase			5000
	β-lipase			4000

* One ml. of an enzyme solution was shaken with 3 ml. of 0.2 *M* phosphate buffer, 7 ml. of water and 2 ml. of olive oil at 30° for one hour. The reaction mixture was titrated with 0.1 *N* alcoholic potassium hydroxide.

lipase therein, and the adsorbent, after being washed well with water, was eluted with 6 liter of 1 *M* ammonium sulfate solution. Ammonium sulfate was dissolved in the eluate, the resultant precipitate was filtered and dissolved in a small amount of distilled water, and the solution was again dialyzed overnight against 0.5 per cent solution of ammonium sulfate. The protein solution was diluted with distilled water to the concentration of 5 mg./ml., treated with 0.45 its volume of saturated ammonium sulfate solution, and

the resultant precipitate was discarded. The solution was then treated with 0.7 its volume of saturated ammonium sulfate solution, and the resultant precipitate was collected, dissolved in distilled water and dialyzed overnight at 2–3° as above. In this way, almost all the lipase in the material could be collected. To purify the lipase further, the aqueous solution of lipase was diluted to the concentration of 5 mg./ml. and subjected to fractionation with ammonium sulfate. In this case, the concentration of the ammonium sulfate solution at each precipitation was increased by 5 per cent from 0.45 to 0.7 its volume of saturated ammonium sulfate solution. In this way, most of the lipase was collected in 60–65 per cent portion. The product was further dialyzed against 0.5 per cent solution of ammonium sulfate at 2–3° and finally lyophilized to give partially purified lipase in powdery form. The yield is 1000 mg. or 14 per cent of the total activity of the filtered broth, and the unit is 2,000 u./mg. (Table I.)

b) Separation of α-, and β-Lipase from Partially Purified Lipase: Both α- and β-lipase were separated from the partially purified lipase by chromatography. The column was produced by packing a mixture consisting of 1.5 g. of calcium phosphate, 4.5 g. of Celite 535 and distilled water in a glass tube

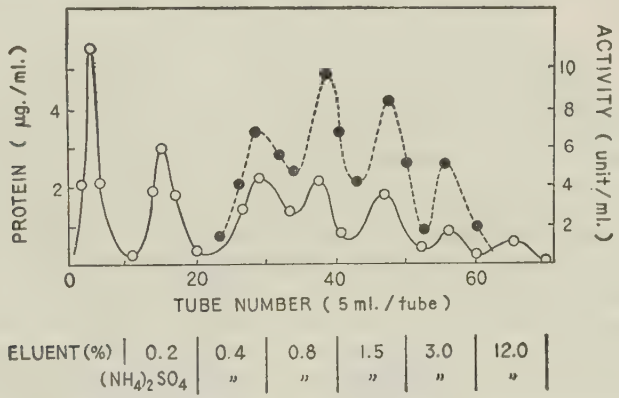


FIG. 1. Chromatography of partially purified lipase. Enzyme was poured through a column of a 1 : 3 mixture of calcium phosphate and Celite, and then eluted with $(NH_4)_2SO_4$-solutions. —○— protein, - -●- - activity.

with a diameter of 1 cm. A solution containing 10 mg. of protein (2,500 u./mg. protein) and a minimum possible amount of salt was poured through the column. The column was washed well with distilled water and then eluted with 50 ml. each of 0.2, 0.4, 0.8, 1.5, 3.0 and 12.0 per cent solutions of ammonium sulfate to give seven fractions of protein. As enzymatic activity was found only in the fractions of 0.4, 0.8, 1.5 and 3.0 per cent ammonium sulfate eluates, they were again chromatographed in the same manner, respectively. In this case, the protein contained in the 0.4, 0.8 and 1.5 per cent ammonium sulfate eluates was eluted only by 1.5 per cent ammonium sulfate solution, and the protein contained in the 3.0 per cent

ammonium sulfate eluate only by 3.0 per cent ammonium sulfate solution. The two proteins thus obtained were again chromatographed separately, but they were eluted only by ammonium sulfate solutions of the same concentrations as above, respectively. This fact showed that the partially purified lipase contained two kinds of lipase, and thus the former was named α-lipase and the latter β-lipase. The yields of α- and β-lipase were 3 mg. (5,000 u./mg. protein) and 2 mg. (4,000 u./mg. protein) respectively.

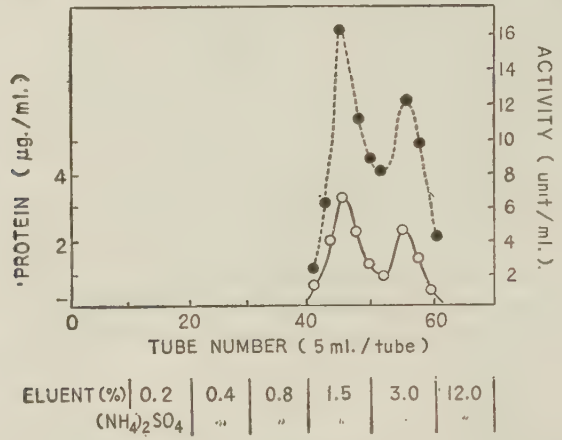


FIG. 2. Rechromatography of purified lipase under the same conditions as Fig. 1. —○— protein, - -●- - activity.

Stability—a) Stability at Various pH's: Ten mg. per ml. solutions of lipase in 0.02 *M* phosphate buffer and 0.02 *M* acetate buffer were left standing at 25° for one hour and the enzymatic activities of the solutions were measured. The enzymes were inactivated atpH lower than 3.5 or higher than 8.5. Both activities were most stable at about 5.8 as was the case with the crude product.

b) Stability at Various Temperatures: A 10 mg. solution of lipase in 0.02 *M* phosphate buffer was left standing at various temperatures for one hour. The enzymatic activity of the solution lowered with rising temperature and disappeared at 50–60°.

c) Stability and Turbidity of the Aqueous Solution of Lipase at Various Salt Concentrations: The aqueous solution of lipase is stable when its activity is lower than 200 u./mg. protein, conversely, it become unstable with increasing activity. This decomposition seems to be prevented in the presence of salt. To confirm this, the following experiment was conducted.

Lipase solutions with various activities were dialyzed against solutions of various salts of various concentrations at 1–3°, and their activities were measured at regular intervals. As seen from Fig. 3, when an aqueous solution of lipase was dialyzed against 0, 5 and 15 per cent ammonium sulfate solutions, the decomposition of the solution was retarded in proportion to the concentration of the salt solutions. It was also observed that when the salt content lowered, an insoluble substance precipitated, which became

no longer soluble by addition of the salt or a concentrate of the dialyzed substance. And the lipase of low activity thus obtained could no longer regain its original activity by addition of salt or a concentrate of the dialyzed substance.

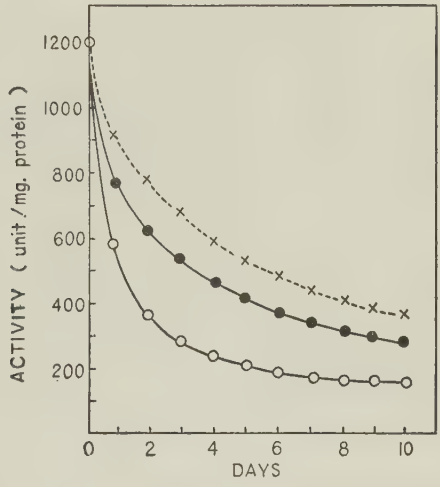


FIG. 3. Stability of aqueous solution of purified lipase at various salt concentrations. One per cent solution of purified lipase (1,300 u./mg. protein) was dialyzed against —○— water, —●— 5 per cent and --×-- 15 per cent ammonium sulfate solutions at 1–3°.

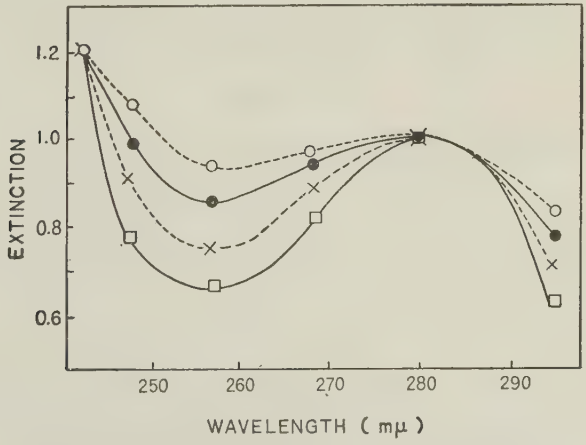


FIG. 4. UV spectra of lipase with various activities, --○-- inactivation, —●— 160 u./mg. protein, --×-- 220 u./mg. protein and —□— 430 u./mg. protein.

d) Effects of Reducing Agents and Metal Ions: When a metal ion such as Fe^{++}, Fe^{+++}, Ca^{++}, Mg^{++}, Co^{++}, Zn^{++}, Sn^{++}, Ni^{++} or Al^{+++} was added in a

concentration of $10^{-3}-10^{-4}$ *M* to a solution of lipase of high activity and to a solution of such a lipase of low activity as obtained in (*c*), the activity of the former was lowered as was the case when no metal ion was added, and the activity of the latter was not heightened. Addition of cystein, L-ascorbic acid, or EDTA exerted no influence.

UV-Absorption of Various Lipases—UV-absorptions of lipase of high activity and of such a lipase of low activity as mentioned above were measured. As shown in Fig. 4, lipase has an absorption maximum at 280 mμ and an absorption minimum at 255 mμ. Observation of UV-absorptions of lipase having 0, 160, 220 and 430^{u}./mg. protein activities, revealed that difference in height between the maximum and the minimum decreases with lowering activity.

Optimum pH of α-, β-, and Partially Purified Lipase—The three preparations of lipase were dissolved in a concentration of 0.005 mg. protein/ml. in buffers of various pH's and relationship between the enzymatic activity and pH was observed. As shown in Fig. 5, it was found that the partially purified lipase has two pH optima at 4.9 and 6.8, while α-lipase and β-lipase have pH optima at 4.9 and 6.8, respectively. From this fact, it is evident that α- and β-lipase are the main components of the partially purified lipase.

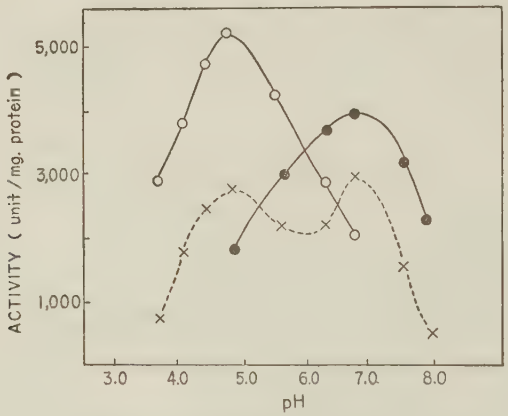


FIG. 5. PH optima of α-lipase, β-lipase and partially purified lipase. —○— α-lipase, —●— β-lipase, - -×- - partially purified lipase.

Studies on the Paper Electrophoresis of α-, β-, and Partially Purified Lipase—The three lipase preparations were subjected to paper electrophoresis according to the usual procedure in various buffers with various pH's at a temperature lower than 5° for 2.5 hours (200–300 v./cm., 2–4 mA./30 cm.). In this case, the partially purified lipase migrated at neutral or alkaline pH. At alkaline pH, it gave four migrated spots, which are probably secondary products and have no enzymatic activity. The partially purified lipase maintained its activity in 0.02 *M* veronal buffer of pH 7.0 and gave two spots, one of

them migrated fast and the other slowly toward the cathode. α-Lipase migrated faster than β-lipase and their speeds were corresponding to those of the above two spots, respectively. This fact also shows that α- and β-lipase are the main components of the partially purified lipase.

SUMMARY

R-302, a strain belonging to *Rhizopus*, was cultivated aerobically and from the filtered broth, partially purified lipase was obtained by fractionation with ammonium sulfate and subsequent adsorption by calcium phosphate. A solution of the product was poured through a column of a 1 : 3 mixture of calcium phosphate and Celite, and then eluted with 0.2, 0.4, 0.8, 1.5, 3.0 and 12 per cent ammonium sulfate solutions, successively. Two protein portions having the activity of lipase were eluted by the 1.5 and 3 per cent ammonium sulfate solutions respectively, and the former was named α-lipase and the latter β-lipase. These lipases are extremely unstable as solution in water but are a little stabilized in the presence of salt. Their aqueous solutions lose activity by half in one hour at 30–40° and become completely inactive in one hour at 60°, and also at pH higher than 8.5. The partially purified lipase has two pH optima at 4.9 and 6.8 and the former corresponds with the pH optimum of α-lipase and the latter with that of β-lipase. Paper electrophoresis of the partially purified lipase gives two spots, one of which migrates fast and the other slowly, and their speeds correspond with those of α- and β-lipase, respectively.

The authors are grateful to Dr. S. Kuwada and Dr. K. Sato for their encouragement throughout this work.

Thanks are also due to members who performed tank culture.

REFERENCES

(*1*) Fiore, J. V., and Nord, F. F., *Arch. Biochem. Biophys.*, **26**, 382 (1950)
(*2*) Peters, I. I., and Nelson, F. E., *J. Bacteriol.*, **55**, 581 (1948)
(*3*) Evans, A. C., *J. Bacteriol.*, **2**, 185 (1917)
(*4*) Matsumura, C., Presented at the Meeting of the Japan Agricultural Chemical Society, April 1956

The Journal of Biochemistry, Vol. 46, No. 5, 1959

AMINO ACID SEQUENCE IN THE N-TERMINAL REGION OF TAKA-AMYLASE A

By AKIRA TSUGITA

(*From the Laboratory of Biochemistry, Faculty of Science, Osaka University, Osaka*)

(Received for publication, September 5, 1958)

Crystallization of Taka-amylase A (TAA) in 1952 in this laboratory (*1*, *2*) first opened a possibility to the systematic investigation of its chemical structure. It has already been reported that alanine is the only one N-terminal amino acid residue of the enzyme protein (*3*), and later work has led to a conclusion that the N-terminal region of the amylase molecule has the sequence, alanyl-glycyl-aspartic acid (*4*).

The purpose of the present paper is to describe experiments undertaken to obtain more detailed information on the structure of the N-terminal part of the amylase protein. For this purpose a relatively large amount of TAA was dinitrophenylated and the resultant dinitrophenylated amylase (DNP-TAA) was subjected to partial hydrolysis. The DNP-peptides derived from the amino terminus were extracted from the hydrolysate and purified by counter-current distribution and other techniques. The amino acid compositions and sequences of the purified peptides were then determined by various analytical methods. It was thus concluded that the following sequence may be assigned to the N-terminal region of TAA; alanyl-glycyl-aspartyl-glutamyl-seryl-alanyl-leucyl-threonine.

EXPERIMENTAL

DNP-TAA—Crystalline TAA was prepared and recrystallized three times by the method of Akabori *et al.* (*1*, *2*). Twenty grams of TAA were dinitrophenylated according to the procedure described by Akabori and Narita (*3*, *4*, *10*) and 22.5 g. of DNP-TAA were obtained. This product contained about fifty moles of DNP groups per mole of TAA, suggesting that not only the N-terminal alanine but also the ε-amino groups of lysyl residues and hydroxyl groups of tyrosyl residues had been dinitrophenylated.

Partial Hydrolysis of DNP-TAA—Twenty grams of DNP-TAA were dissolved in 500 ml. of freshly distilled 80 per cent formic acid and heated for 5 hours in a boiling water bath (*4*). The reaction mixture was concentrated *in vacuo* to about 50 ml. below 45°. In order to remove formic acid completely, water was occasionally added to the mixture during the evaporation.

Extraction of DNP-Peptides—The concentrated hydrolysate was diluted to 1,000 ml. with water and acidified with 2 *N* hydrochloric acid. After removing insoluble larger peptides by centrifugation, the solution was extracted with ethyl acetate. The ethyl acetate layer was washed with water and treated with three portions of aqueous sodium bicarbonate

solution. The combined bicarbonate extracts were re-extracted with ethyl acetate. The yellowish extract containing DNP-peptides was evaporated to dryness.

Fractionation of DNP-Peptides—A 300-tubes counter-current distribution apparatus constructed in this laboratory (*5*) was mainly used for the fractionation of DNP-peptides. Prior to distribution, most of the contaminated dinitrophenol was removed by the cold finger technique (*15*). The solvent system used was suggested from the work of Craig (*6*) and had the composition; chloroform, acetic acid and 0.1 *N* hydrochloric acid (2 : 2 : 1 by volume). The distribution curves were drawn photometrically measuring the amounts of DNP-compounds in each tube at 360 mμ.

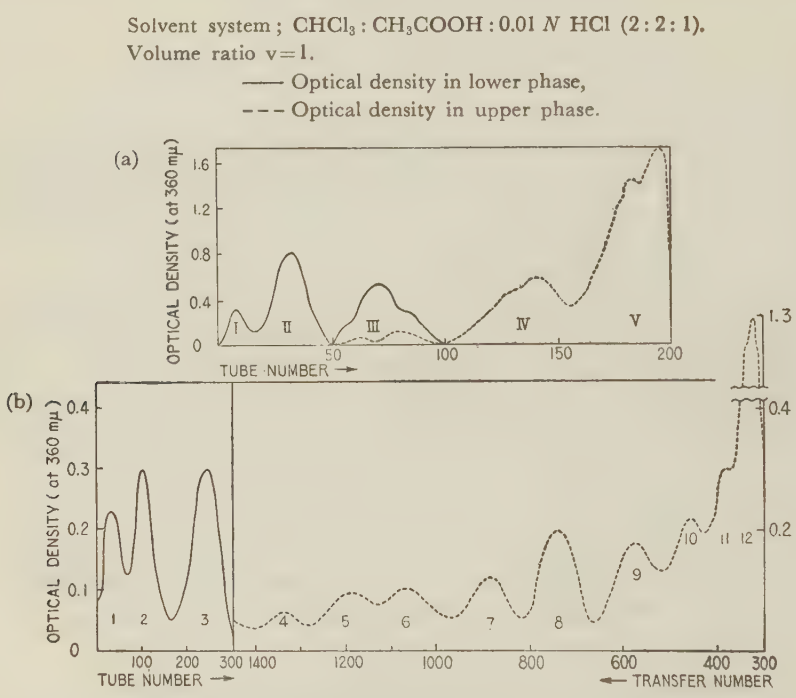


FIG. 1. Countercurrent distribution curve of N-terminal peptides in Taka-amylase A.

Fig. 1-a shows the distribution curve obtained after 200 transfers in fundamental procedure with a volume factor of 1 (v=1). Since celite and silica gel column chromatography revealed that each peak in this curve still contained several components, distribution was further continued by another 100 transfers under the same conditions followed by 1150 single withdrawal procedures, the volume factor being changed to 0.5 (v=0.5). The distribution curve obtained after these procedures is illustrated in Fig. 1-b. Peaks in this curve were still found to contain more than one component (see Fig. 2). Further

fractionation was therefore performed either by redistribution in the same apparatus or by chromatographic techniques.

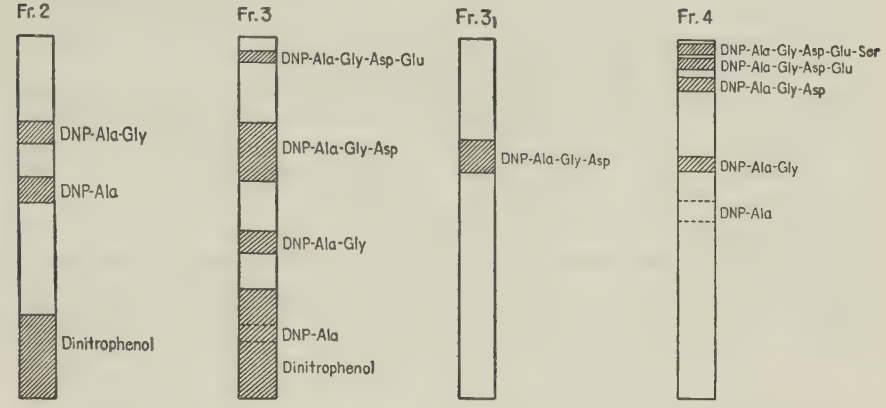


FIG. 2. Isolation of DNP-peptides by column chromatography.

Amino Acid Compositions of DNP-Peptides—The amino acid compositions of sufficiently purified DNP-peptides were determined after dinitrophenylation of the acid hydrolysates by the method of Koch (*7*) or Levy (*8*). In some critical cases, the ion-exchange chromatographic analysis of Moore and Stein (*9*) was also employed.

N-Terminal Residue of DNP-Peptides—Although it was evident from previous work (*3*, *4*) that the N-terminal amino acid residue of all the N-terminal DNP-peptides was alanine, this was further confirmed in each peptide by the two-dimensional paper chromatography (*7*, *8*).

C-Terminal Residue of DNP-Peptides—This was determined by the hydrazinolysis method developed in this laboratory (*11*). The sample to be examined was dissolved in a small amount of acetone, placed in a decomposition vessel and the acetone was evaporated off *in vacuo*. A few drops of anhydrous hydrazine were added to the residue and the mixture was maintained at 100° for 6 hours. The reaction mixture was dried *in vacuo* at room temperature and the residue was dinitrophenylated by the usual method. The DNP-amino acids obtained from C-terminus and DNP-derivatives obtained from acidic amino acid monohydrazides were extracted with 2 per cent aqueous sodium bicarbonate from ether solution. After acidification of the aqueous layer, they were re-extracted with ethyl acetate. The determination of DNP-derivatives was carried out by the chromatographic techniques as described above. All the yellow bands were taken out and subjected to hydrolysis with 6 *N* HCl at 100° for 6 hours. In the hydrolysis DNP-amino acid remained unchanged, but DNP-derivatives of mono-hydrazide of glutamic or aspartic acid were converted to the corresponding DNP-amino acids.

Other Technique Employed—For separation and/or identification of a number of DNP-peptides, DNP-amino acids and free amino acids, several types of chromatography were employed. Paper chromatography, both one- and two- dimensional, of free amino acids was

conducted with 80 per cent aqueous phenol or acetic acid-*n*-butanol-water (1 : 4 : 1) as solvents. For paper chromatography of DNP-derivatives 1.5 *M* phosphate buffer of pH 6.5 (*8*) or *n*-butanol saturated with aqueous ammonia (*7*) was used as a solvent. Ethyl acetate-phosphate buffer of various pH was used for celite column chromatography (*12*), and chloroform-*n* (or *sec*)-butanol-aqueous acetic acid for silica gel column chromatography (*13*). Both types of column were used for DNP-peptides.

In several instances, paper electrophoresis was also used for the separation of DNP-peptides. The size of paper strips was 30 × 1.0 cm. and the voltage gradient applied was about 10–15 per cm. (0.5 to 1.0 mA. per cm. in section). The buffer used was acetic acid-pyridine which was prepared by adding pyridine to *M*/6 acetic acid until the pH became 6.5.

Amino Acid Sequences of DNP-Peptides—The amino acid sequences of some of the DNP-dipeptides were determined by direct comparison on paper chromatograms with the authentic samples. For the majority of purified DNP-peptides, however, the sequences were concluded from their amino acid compositions, N- and C-terminal residues, and other available information obtained by more than two analytical techniques described above.

RESULTS

As described above, peaks in the distribution curve of the original mixture of DNP-peptides (Fig. 1-b) were not yet homogeneous and had to be further fractionated to obtain pure fractions. Peaks 2, 3 and 4 in Fig. 1-b which still contained dinitrophenol and other interferring substances were therefore again distributed in the countercurrent distribution apparatus using a mixture of benzene, acetic acid and 0.1 *N* hydrochloric acid (2 : 2 : 1

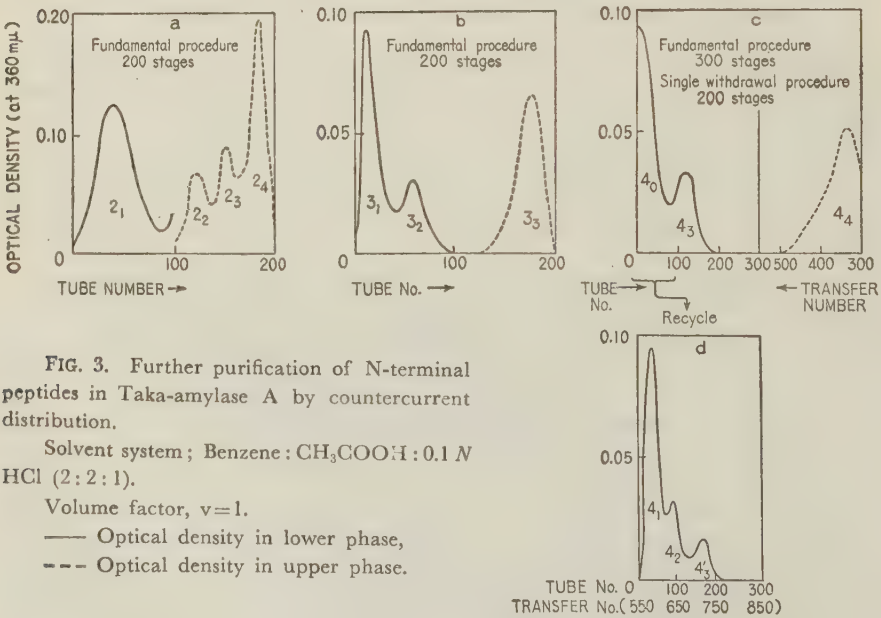


FIG. 3. Further purification of N-terminal peptides in Taka-amylase A by countercurrent distribution.

Solvent system; Benzene : CH_3COOH : 0.1 *N* HCl (2 : 2 : 1).

Volume factor, v = 1.

—— Optical density in lower phase,

- - - Optical density in upper phase.

by volume); the volume factor being 1 (v=1). The distribution curves thus obtained are shown in Fig. 3–a, b and c.

As can be seen from Fig. 3a, Peak 2 was separated into four fractions; among them Peaks 2_1 and 2_2 were identified as DNP-alanyl-glycine and DNP-alanine, respectively, by chromatographic comparison with the authentic samples. Peak 2_3 seemed to contain dinitrophenol and a still unidentified compound, while the component in Peak 2_4 was not yet determined. At any rate, it seemed likely that the main components in Peak 2 were DNP-alanine and DNP-alanyl-glycine.

Fig. 3-b shows that Peak 3 consisted of three components, of which Peak 3_2 and 3_3 were identified as DNP-alanyl-glycine and dinitrophenol, respectively. The component in Peak 3_1 was found to be a DNP-tripeptide consisting of alanine, glycine and aspartic acid; the C-terminus being aspartic acid. It was therefore identified as DNP-alanyl-glycyl-aspartic acid.

As shown in Fig. 3-c, Peak 4 was also fractionated into three fractions, *i.e.*, Peaks, 4_0, 4_3 and 4_4. Peaks 4_3 and 4_4 were found to contain DNP-alanyl-glycine and dinitrophenol, respectively. Peak 4_0 was, however, not yet homogeneous. The content of tubes corresponding to Peaks 4_3 and 4_4 were then replaced by the freshly prepared solvent adding no more upper phase and distribution was continued by recycling procedure one time. Peak 4_0 was thus separated into three components, *i.e.* Peaks 4_1, 4_2 and $4_3'$, as shown in Fig. 3-d. From the amino acid composition and N- and C-terminal analyses of these components they were identified as DNP-alanyl-glycyl-aspartyl-glutamic acid, DNP-alanyl-glycyl-aspartic acid and DNP-alanyl-glycine, respectively. As can be seen from Fig. 3-d, Peaks 4_1 and 4_2 were rather asymmetric indicating that the components in these peaks might have been partly decomposed during the distribution procedures.

Since Peaks 5 to 11 appeared to contain ε-DNP-lysyl peptides, they were subjected to paper electrophoresis in order to remove these ε-DNP-lysyl

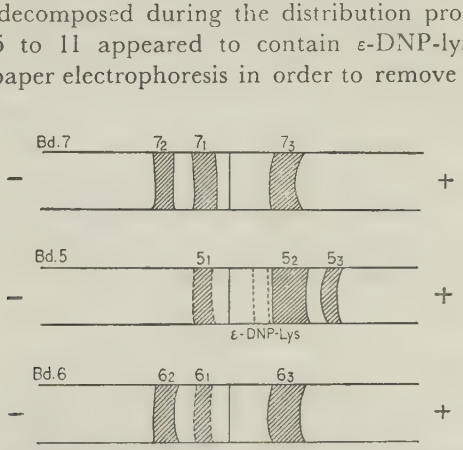


FIG. 4. Separation of DNP-peptides by paper electrophoresis.
Buffer; Pyridine: acetic acid: H_2O (100 : 10 : 890) pH 6.5.
Filter paper; Toyo-Roshi No. 50.
Conditions; 10～15 v./cm. 5～10 hours.

peptides from N-terminal DNP-peptides. Typical electrophoretic patterns obtained are illustrated in Fig. 4.

As can be seen from Fig. 4, N-terminal DNP-peptides migrated toward the cathode, while ε-DNP-lysyl-peptides remained at the starting line or slightly moved toward the anode. After electrophoresis, the yellow bands corresponding to the terminal peptides were cut off and eluted at 60° with 50 per cent aqueous acetone which had been made slightly alkaline with a few drops of ammonia.

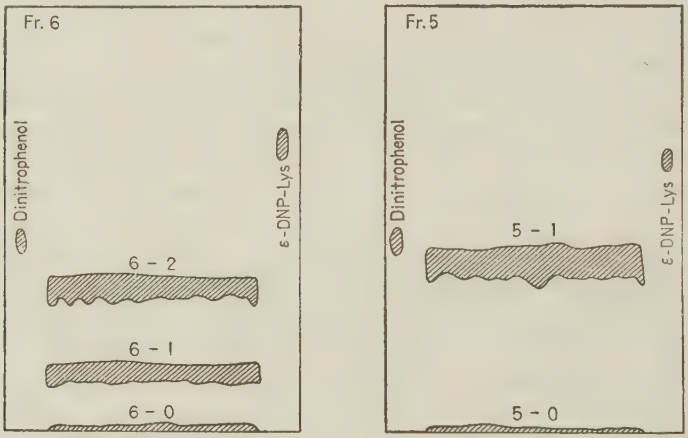


FIG. 5. Separation of DNP-peptides by one-dimensional paper chromatography. (Solvent: 1.5 *M* phosphate buffer pH 6.5, filter paper: Toyo-Roshi No. 51)

The terminal DNP-peptide fractions thus separated were not yet sufficiently pure. They were therefore further purified by chromatographic methods such as celite and silica gel column chromatography (Fig. 2) and one-dimensional paper chromatography (Fig. 5). A suitable method was chosen for each fraction according to its quantity, solubility and purity. Most of the DNP-peptides thus purified were then analysed for their amino acid compositions and N- and C-terminal residues. The amino acid sequences in these peptides were inferred from these and other findings.

The results of the sequential analyses of N-terminal DNP-peptides just described may be summarized as shown in Table I.

DISCUSSION

The previous report from this laboratory (*4*) has shown that the N-terminal region of TAA has the sequence of alanyl-glycyl-aspartic acid. This was reconfirmed in the present work. The results recorded in Table I show that the next amino acid residue is in all probability glutamic acid. This conclusion was also supported by an independent investigation using a crystalline protease obtained from *Bacillus subtilis* (*14*) as a tool, revealing

the sequence, alanyl-glycyl-aspartyl-glutamic acid, as the N-terminal structure of TAA (*16*).

The next longer DNP-peptide to which the hydrazinolysis method could assign a definite C-terminal residue was one of the component found in peak 7, Peak 7_2, and its C-terminus was determined to be alanine. Although this finding alone seemed to suggest that an alanyl residue occurs next to the glutamyl residue, it was concluded that the sequence alanyl-glycyl-aspartyl-glutamyl-seryl-alanine should be preferred to the sequence -glutamyl-alanine for the real structure of the N-terminal region of TAA. This conclusion was based upon the following two lines of evidence. 1) The amino acid analysis of this peptide revealed the presence of serine among its component amino acids in addition to one mole of alanine. 2) Though the hydrazinolysis of the DNP-peptide in Peak 6 gave no clear-cut result in the determination of the C-terminal residue, serine was detected in the lysate with small quantities of other amino acids such as glutamic acid, aspartic acid and glycine. Therefore it seemed likely that the structure of this peptide was DNP-alanyl-glycyl-aspartyl-glutamyl-serine.

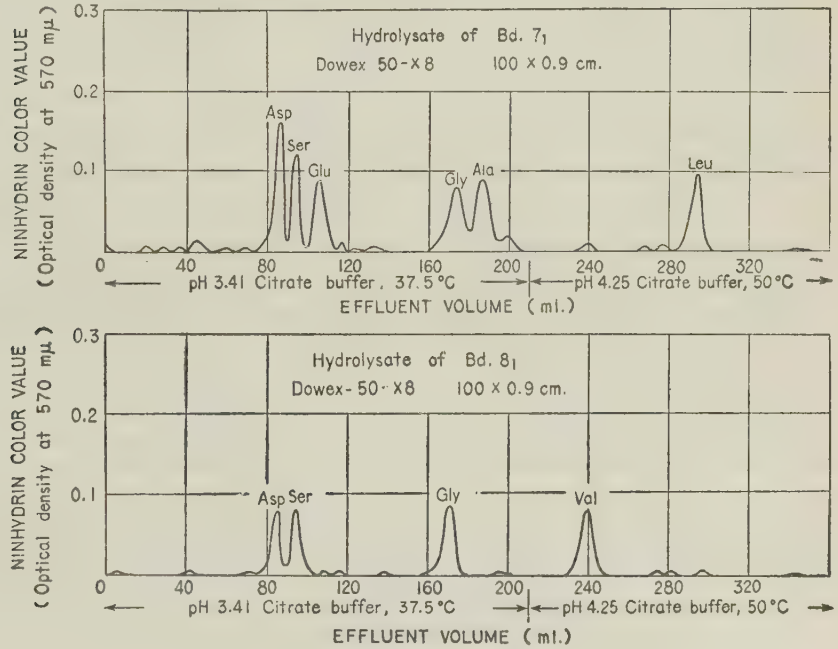


FIG. 6. Column chromatogram of amino acid composition.

The linkage between the glutamyl and seryl residues appears to be rather unstable, and such lability seems to be mainly responsible for the difficulty encountered in its purification. The peptide might have been partly decomposed during the counter-current distribution procedure as judged from the asymmetric shape of the peak obtained after distribution.

Investigations on the other component present in Peak 7, Peak 7_1, suggested that the amino acid occurring next to the second alanyl residue was leucine (Fig. 6 and Table I).

TABLE I

Investigation of Amino Acid Composition and N- and C-terminal in N-terminal Region in TAA

Peak No.	N-terminal (DNP-)	Composed amino acid	C-terminal	Amino acid sequence
2_3	DNP-OH			
2_2	Ala			Ala
2_1	Ala	Gly	Gly	Ala-Gly
3_3	Ala DNP-OH			Ala
3_2	Ala	Gly	Gly	Ala-Gly
3_1	Ala	Gly, Asp	Asp	Ala-Gly-Asp
4_3	Ala	Gly	Gly	Ala-Gly
4_2	Ala	Gly, Asp	Asp	Ala-Gly-Asp
4_1	Ala	Gly, Asp, Glu	Glu	Ala-Gly-Asp-Glu
6_2	Ala	Gly, Asp, Glu (Ser)	Ser (Glu, Asp, Gly)	Ala-Gly-Asp-Glu-Ser
7_2	Ala	Gly, Asp, Glu, Ser, Ala	Ala	Ala-Gly-Asp-Glu-Ser-Ala
7_1	Ala	Gly, Asp, Glu, Ser, Ala, Leu	Leu	Ala-Gly-Asp-Glu-Ser-Ala-Leu
9_2	Ala	Gly, Asp, Glu, Ser, Ala, Leu (Thr)	Thr (Leu)	Ala-Gly-Asp-Glu-Ser-Ala-Leu-Thr
11	Ala	Gly, Asp_2 Glu, Ser_2 Ala, Leu, Thr, Val_2 Phe, Lys*	—	Ala-Gly-Asp-Glu-Ser-Ala-Leu-Thr-(Val_2Asp, Ser, Phe, Lys)
5_2		Lys* Asp, Glu		(Lys, Asp, Glu)
5_3		Lys* Glu, Ala		(Lys, Glu, Ala)
6_3		Lys* Asp, Phe, Tyr**		(Lys, Asp, Phe, Tyr)
7_3		Lys* Asp, Glu, Leu, Arg		(Lys, Asp, Glu, Leu, Arg)
8_0		Lys*		Lys
8_3	Val	Lys* Asp, Gly, Ser		Val-(Lys, Asp, Gly, Ser)
8_1	Val	Lys* Asp, Gly, Ser, Phe		Val-(Lys, Asp, Gly, Ser, Phe)

Lys*; ε-DNP-Lys, Tyr**; *O*-DNP-Tyr.

Extremely small quantities of peptides which are longer than DNP-alanyl-glycyl-aspartyl-glutamyl-seryl-alanyl-leucine seem to be present in the partial hydrolysate of the original DNP-protein. Careful study of a minute amount of DNP-peptide purified from Peak 9, however, permitted the qualitative identification of amino acid linked to the leucyl residue as threonine.

Peak 11 was also fractionated by the techniques of paper electrophoresis and paper chromatography. One of the peptide in Peak 11 (Peak 11_2) contained two moles of valine, each one mole of aspartic acid, serine, phenylalanine and lysine besides N-terminal amino acid, DNP-alanine, and the amino acid constituent of Peak 9_2 (Table I). But as this fraction obtained was very small, the determination of C-terminal or other investigation could not be conducted. It was suggested that the further structure of N-terminal region was alanyl-glycyl-aspartyl-glutamyl-seryl-alanyl-leucyl-threonyl-(aspartic acid, serine, valine 2, phenylalanine, lysine)-.

To sum up the experimental results and discussions described above, it may be concluded that the N-terminal part of the amylase protein has the sequence alanyl-glycyl-aspartyl-glutamyl-seryl-alanyl-leucyl-threonine. It must, however, be pointed out that it is still undecided whether the aspartyl or glutamyl residue or the both are in the amide forms in the native protein.

Preliminary experiments were also conducted with non-terminal peptides containing ε-DNP-lysine which were separated from the N-terminal DNP-peptides by paper electrophoresis. As is summarized in Table I, six such peptides were purified and analysed. They are valyl-(glycine, aspartic acid, serine and ε-DNP-lysine), valyl-(glycine, aspartic acid, serine, phenylalanine and ε-DNP-lysine) from Peak 8, (aspartic acid, glutamic acid, arginine, leucine and ε-DNP-lysine) from Peak 7_3, (aspartic acid, O-DNP-tyrosine, phenylalanine and ε-DNP-lysine) from Peak 6_3, and (aspartic acid, glutamic acid and ε-DNP-lysine) and (alanine, glutamic acid and ε-DNP-lysine) from Peak 5_2 and Peak 5_3, respectively.

A brief mention may be added concerning the conditions of partial hydrolysis of the DNP-protein. It was reported in the previous paper (*4*), that the dipeptide DNP-alanyl-glycine was the only peptidic product obtained when DNP-TAA was hydrolysed with mineral acids even under mild conditions. In the present investigation, therefore, the hydrolysis was carried out with 85 per cent formic acid as in the previous report (*4*). The hydrolysis conditions employed in this investigation appear to favor for the production of large amounts of tetra- and penta-peptides. The extraction of the N-terminal DNP-peptides from the hydrolysate was performed with ethyl acetate, although this solvent does not seem to be quite suitable for the purpose in view of the fact that it also extracts considerable quantities of ε-DNP-lysyl and O-DNP-tyrosyl peptides, it is definitely superior to ether which is unable to extract desired longer peptides.

SUMMARY

TAA was dinitrophenylated and partly hydrolysed with 85 per cent formic acid. The N-terminal DNP-peptides were extracted from the hydrolysate and separated from each others by means of countercurrent distribution method and other kinds of techniques. The purified DNP-peptides were

then analysed for amino acid compositions, N- and C- terminal residues, etc. From these analytical experiments, it was concluded that the amino acid sequence in the N-terminal region of TAA is alanyl-glycyl-aspartyl-glutamyl-seryl-alanyl-leucyl-threonine.

The author wishes to express his gratitude to Prof. S. Akabori for his kind guidance, and to Dr. T. Ikenaka for his helpful advice during the course of this investigation, and also to Sankyo Co., Ltd. for their kind supply of "Takadiastase Sankyo".

REFERENCES

(*1*) Akabori, S., Hagihara, B, and Ikenaka, T., *Proc. Japan. Acad.*, **27**, 350 (1951)
(*2*) Akabori, S., Ikenaka, T., and Hagihara, B., *J. Biochem.*, **41**, 577 (1954)
(*3*) Akabori, S., and Ikenaka, T., *J. Biochem.*, **42**, 603 (1955)
(*4*) Narita, K., and Akabori, S., *J, Bioch m.*, **46**, 91 (1959)
(*5*) Akabori, S., Tani, H., and Tsugita, A., *Japan Analyst*, **4**, 119 (1952)
(*6*) Hausmann, W., Weisiger, J. R., and Craig, L. C., *J. Am. Chem. Soc.*, **77**, 724 (1955)
(*7*) Koch, G., and Weidel, W., *Z. physiol. Chem.*, **303**, 213 (1956)
(*8*) Levy, A. L., *Nature*, **174**, 126 (1954)
(*9*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192**, 663 (1951)
(*10*) Sanger, F., *Biochem. J.*, **39**, 507 (1945)
(*11*) Akabori, S., Ohno, K., Ikenaka, T., Okada, Y., Hanafusa, H., Haruna, I., Tsugita, A., Sugae, K., and Matsushima, T., *Bull. Chem. Soc. Japan*, **29**, 507 (1956)
(*12*) Perrone, J. C., *Nature*, **167**, 513 (1951)
(*13*) Tristram, G. R., *Biochem. J.*, **40**, 721 (1946)
(*14*) Hagihara, B., Matsubara, H., Nakai, M., and Okunuki, K., *J. Biochem.*, **45**, 185 (1958)
(*15*) Mills, G. L., *Biochem. J.*, **50**, 707 (1952)
(*16*) Akabori, S., and Tsugita, A., to be published

The Journal of Biochemistry, Vol. 46, No. 5, 1959

STUDIES ON THE AFFINITY OF HEME TO PROTEINS

By AKIHIKO KAJITA, FUJIKO UCHIMURA, HITOSHI MIZUTANI
GORO KIKUCHI and KOOZOO KAZIRO

(*From the Biochemical Laboratory, Nippon Medical School, Tokyo*)

(Received for publication, September 11, 1958)

As is well known, when hemoglobin is denatured by addition of alkali and sodium dithionite, hemoglobin converts into "alkaline denatured globin hemochrome" in which the 5th and 6th coordination positions of the heme are saturated most probably with the imidazole groups of histidine.

This alkaline denatured globin hemochrome is known to combine with as much as 24 hemes newly added, as previously reported by Holden (*1*) and Horiguchi (*2*). This fact seems to imply the participation of certain groups other than histidine in heme linkage. Further, it was also reported semiquantitatively (*2*) that the affinity of heme linked groups in hemoglobin seems to be much higher than that of free histidine. It is of great interest to explain the marked difference between their heme-combining affinities.

Present authors intended to study more in detail the combining reaction of heme to free histidine, alkaline denatured proteins and proteinase digested hemoglobin.

EXPERIMENTAL

Materials—Crystalline hemin was prepared by the method of Chu and Chu (*3*) and recrystallized from $CHCl_3$-pyridine (*4*). Before use, hemin was dissolved in about 2 ml. of 0.1 *N* NaOH and diluted up to 100 ml. with buffer solutions or distilled water.

Reagent grade L-histidine monohydrochloride was furnished by Wako Jyunyaku Co. Horse oxyhemoglobin was crystallized by Heidelberger's method with a little modification (*5*). Human serum γ-globulin was prepared by Cohn's ethanol fractionation method (*6*). Horse serum albumin was crystallized by salting out method (*7*). Bovine serum albumin was a preparation of Armour Co. Pepsin and trypsin powder supplied by Merck Co. were used without further purification.

Methods and Apparatus—Concentration of each protein solution was estimated by the micro-Kjeldahl method except that hemoglobin was determined by the extinction at 558 mμ as alkaline denatured globin hemochrome.

Combination of hematin to histidine: Two ml. of varied concentrations of neutralized histidine and 1 ml. of buffer were added to 1 ml. of 2.5×10^{-5} *M* hematin solution. Optical density was measured after 5 minutes.

Combination of heme to alkaline denatured proteins: One ml. of varied concentrations of hematin and 3 ml. of 2 *N* NaOH were added to 1 ml. of 3.33×10^{-6} *M* horse oxyhemoglobin. The reaction mixture was immediately reduced by a minimum amount of sodium dithionite and covered with liquid paraffin. After the completion of alkaline

hemochrome formation on standing for 20 minutes, absorption spectra were measured.

Digestion: Digestion with pepsin was carried out at 37°. To 40 ml. of 1.7 per cent protein solution, 10 ml. of HCl and 10 ml. of 50 mg./dl. pepsin were added. Aliquots of reaction mixture were subjected to the succeeding tests at a definite time interval. Hydrogen ion concentration of reaction mixture was adjusted by use of varied concentrations of HCl.

Combination of heme to digest: Two ml. of above described reaction mixture was diluted up to 100 ml., with 0.1 *N* NaOH. To 1 ml. of resulting diluted solution, 2 ml. of 2 *N* NaOH, 1 ml. of 1×10^{-4} *M* hematin, 1 ml. of water and a minimum amount of sodium dithionite were added. After the formation of alkaline hemochrome was in completion, standing for 20 minutes, optical density at 558 mμ was measured.

Biuret test: Three ml. of above mentioned digestion mixture was deproteinized by addition of 5 ml. of 20 per cent trichloroacetic acid. Two ml. of resulted filtrate was treated with 2 ml. of 2 *N* NaOH and 1 ml. of 1 per cent copper sulphate. Precipitate of copper hydroxide was filtered off and the optical density of the filtrate was read at 540 mμ.

Folin's test: One ml. of above noted trichloroacetic acid filtrate was diluted to 10 ml. with water. Five ml. of this solution, 10 ml. of 0.5 *N* NaOH and 3 ml. of three-fold diluted phenol reagent were mixed, and the developed color was estimated at 720 mμ after 5 minutes.

Optical determination was made by use of EPV Model II photoelectric spectrophotometer of Hitachi Co. Ltd. and a cuvette of 10 mm. depth. Hydrogen ion concentration was measured by a glass electrode pH meter of Toyo Rikagaku Co. Ltd.

All the experiments were carried out at room temperature of 20–22°, except where stated otherwise.

RESULTS

I. Combination of Hematin to Histidine—On addition of histidine to hematin solution, the absorption spectra of hematin converted to those of typical hemichrome, characterized by its absorption maxima at 558, 530, and 410 mμ. Fig. 1 shows the rise of the Soret band, accompanied by the increase of histidine. The presence of an isosbestic point at 391 mμ throughout the absorption change indicates that the equilibrium system is established by the two components only, namely hematin and histidine hemichrome. Thus, the existence of any of the intermediate compounds such as monohistidine-hematin may be excluded. From the absorption change at 410 mμ, where the difference in optical density of these two components is maximum, dissociation degree α was obtained. The plots of α against logarythmic concentration of histidine added coincided well with a theoretical curve of second order dissociation.

The plots obtained with varied pHs are summarized in Fig. 2. In these cases, α was plotted against the amounts of histidine added instead of free histidine, since the histidine concentration applied was far greater than hematin concentration so that the amount of bound histidine was negligebly small.

Based on these facts the following formula was presented for the equilibrium system:

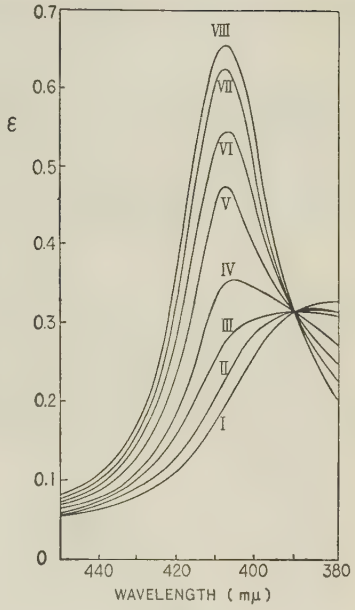


FIG. 1. Changes in absorption spectrum of hematin at the Soret band, on addition of histidine.

Hematin: $6.25\times10^{-6}\,M$. Histidine: I, $0\,M$; II, $1.4\times10^{-2}\,M$; III, $2.0\times10^{-2}\,M$; IV, $2.7\times10^{-2}\,M$; V, $4.1\times10^{-2}\,M$; VI, $5.5\times10^{-2}\,M$; VII, $8.0\times10^{-2}\,M$; VIII, $1.4\times10^{-1}\,M$. pH: 9.0.

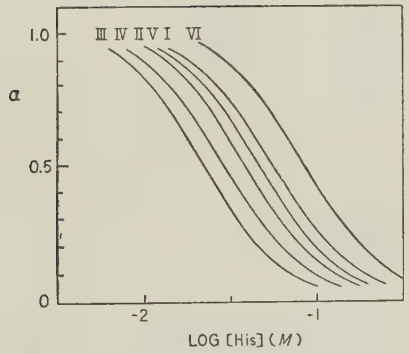


FIG. 2. pH dependence of dissociation curve of histidine hemichrome.

Hematin: $6.25\times10^{-6}\,M$. pH; I, 6.0; II, 6.25; III, 7.05; IV, 9.0; V, 9.3; VI, 10.2.

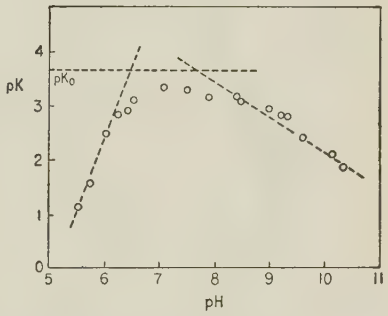


FIG. 3. Relation between pK of histidine hemichrome and pH.

○: pK value observed.

$$\text{Fe-His}_2 \overset{K}{\rightleftarrows} \text{Fe} + 2\ \text{His} \qquad (1)$$

where Fe, His, and Fe-His_2 represent hematin, histidine, and histidine hemichrome, respectively. K is the dissociation constant, which is denoted by the following equation;

$$K = \frac{[\text{Fe}]\,[\text{His}]^2}{[\text{Fe-His}_2]} \qquad (2)$$

In Fig. 3, the pK values calculated from the above equation were plotted

versus pH. The value declines in the acidic and alkaline range with a slope of +2 and −1, respectively, while in the neutral range it remains relatively constant. According to the rule (10) by Dixon (*8*), it may be deduced that the change of pK in function of pH in this case is due to the dissociation of free histidine and hematin, since the data give a curve that is convex upwards.

In histidine molecule, imidazole-NH is reported to dissociate as a weak acid with pK of 6.34 according to the following equation, (*9*).

$$\text{His-H} \underset{}{\overset{K_a}{\rightleftarrows}} \text{His}^- + \text{H}^+ \tag{3}$$

$$K_a = \frac{[\text{His}^-]\,[\text{H}^+]}{[\text{His-H}]} \tag{4}$$

On the other hand, hematin-iron ionizes as a weak base and the absorption spectra change with pH, as shown in Fig. 4. The relation between the

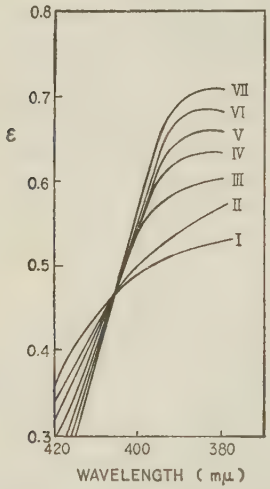


FIG. 4. Effect of pH upon the absorption of Soret band of hematin. Hematin: 1.25×10^{-5} M. pH: I, 4.5; II, 6.0; III, 6.98; IV, 7.75; V, 8.0; VI, 8.52; VII, 10.1.

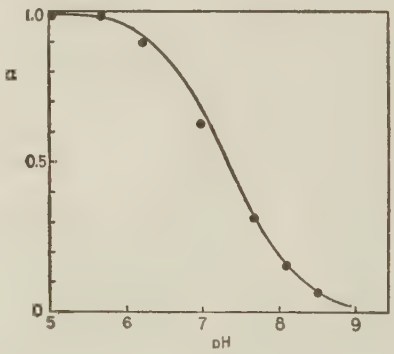


FIG. 5. Dissociation curve of hematin iron. Solid circles: estimated value. Full curve: theoretical dissociation curve.

dissociation degree and pH is plotted in Fig. 5. The plots were in good agreement with a theoretical dissociation curve of first order and its pK was found to be 7.3. Clark *et al.* have reported the pK value of proto-hematin to be 7.6 (*10*).

The dissociation equilibrium of hematin iron is represented as follows:

$$\text{Fe-OH} \underset{}{\overset{K_b}{\rightleftarrows}} \text{Fe}^+ + \text{OH}^- \tag{5}$$

$$K_b = \frac{[\text{Fe}^+]\,[\text{OH}^-]}{[\text{Fe-OH}]} \tag{6}$$

where Fe-OH and Fe^+ represent hematin hydroxide and acid hematin.

By combining equations (3), (5) and (1), we obtain:

$$\begin{array}{ccc} \text{Fe-His}_2 & \overset{K_0}{\rightleftarrows} & \text{Fe}^+ + 2\ \text{His}^- \\ K_b \uparrow\downarrow & & K_a \uparrow\downarrow \\ \text{Fe-OH} & & 2\ \text{His-H} \end{array} \tag{7}$$

where K_0 is the intrinsic dissociation constant which is defined as follows.

$$K_0 = \frac{[\text{Fe}^+]\,[\text{His}^-]^2}{[\text{Fe-His}_2]} \tag{8}$$

In this case, it is assumed that the combination of histidine to hematin occurs independently of the ionization of other groups than imidazole-NH and hematin iron. Rearrangement of equations (2), (4) and (6) gives:

$$K = \frac{[\text{Fe}]\,[\text{His}]^2}{[\text{Fe-His}_2]} = \frac{[\text{Fe}^+ + \text{Fe-OH}]\,[\text{His}^- + \text{His-H}]^2}{[\text{Fe-His}_2]} \tag{9}$$

$$[\text{His-H}] = \frac{[\text{His}^-]\,[\text{H}^+]}{K_a} \tag{10}$$

$$[\text{Fe-OH}] = \frac{[\text{Fe}^+]\,[\text{OH}^-]}{K_b} = \frac{[\text{Fe}^+]\,K_b'}{[\text{H}^+]} \tag{11}$$

$$(K_b' = K_w/K_b,\ K_w = [\text{H}^+]\,[\text{OH}^-])$$

Substituting equations (10) and (11) into equation (9), we have

$$\begin{aligned} K &= \frac{[\text{Fe}^+]\,[\text{His}^-]^2}{[\text{Fe-His}_2]} \cdot (1 + [\text{H}^+]/K_a)^2 \cdot (1 + K_b'/[\text{H}^+]) \\ &= K_0 \cdot (1 + [\text{H}^+]/K_a)^2 \cdot (1 + K_b'/[\text{H}^+]) \end{aligned} \tag{12}$$

or in logarythmic form:

$$pK = pK_0 - 2\log(1 + [\text{H}^+]/K_a) - \log(1 + K_b'/[\text{H}^+]) \tag{13}$$

In this equation, K_a and K_b' are $10^{-6.34}$ and $10^{-7.3}$, respectively. When pH is lower than 4.34,

$$[\text{H}^+]/K_a \gg 1, \quad K_b'/[\text{H}^+] \ll 1$$

Therefore, equation (13) can be simplified as follows:

$$pK = pK_0 + 2\ \text{pH} - 2\ pK_a \tag{14}$$

At pH 6.34,

$$[\text{H}^+]/K_a = 1, \quad K_b'/[\text{H}^+] \fallingdotseq 1/10$$

therefore, equation (13) is rewritten as follows,

$$pK = pK_0 - 2\log 2 - \log 1.1 \tag{15}$$

At pH 7.3,

$$[\text{H}^+]/K_a \fallingdotseq 1/10, \quad K_b'/[\text{H}^+] = 1$$

hence,

$$pK = pK_0 - 2\log 1.1 - \log 2 \tag{16}$$

Above pH 9,

$$[\text{H}^+]/K_a \ll 1, \quad K_b'/[\text{H}^+] \gg 1$$

therefore,

$$pK = pK_0 - \text{pH} + pK_b' \tag{17}$$

From equation (14)~(17), it is well explained that pK increases at lower pH range with a slope of +2 and decreases at higher pH range with a slope of −1, whereas it remains relatively constant at the neutral range. pK_0 calculated from pK at various pH, was 3.6 on an average. In Fig. 3, the slopes of the curve at either side were extrapolated to pK_0. The intercepts were pH 6.4 and 7.7, respectively. These values are very close to pK_a (6.34) and pK_b' (7.3). These facts may be regarded as evidence for the validity of the concepts that the combination of histidine to hematin is followed by a second order dissociation, and that the change of pK is due to the dissociation of imidazole-NH of histidine at lower pH range and of hematin iron at higher pH range.

An attempt was also made to investigate the affinity of ferrous heme for histidine. However, on addition of sodium dithionite, absorption of resulted histidine hemochrome decrease gradually. This tendency was accelerated by aeration and a new absorption maximum appeared at 600 mμ. The fact may be interpreted as indicating that the instability of histidine hemochrome is due to the decomposition of heme to an unknown green pigment, which is different from verdohemochrome. Further precise quantitative study was, therefore, given up. However, the results of semiquantitative observations indicated that the affinity of ferrous heme for histidine may be almost the same as that of hematin.

II. Combination of Heme to Alkaline Denatured Proteins—On addition of heme to alkaline denatured globin hemochrome, the absorption in green region increased as shown in Fig. 6. In Fig. 7 the optical density at 558 mμ are plotted against the quantity of added heme; curve I in this figure is

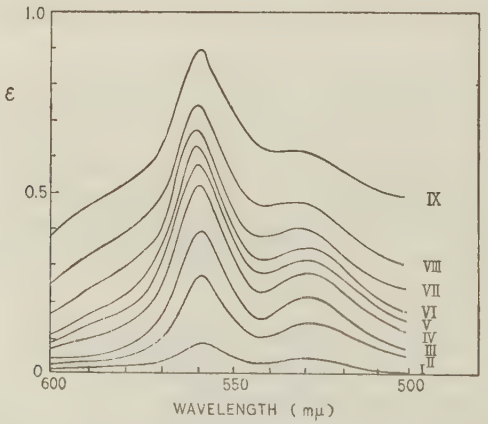


FIG. 6. Absorption changes of alkaline denatured globin hemochrome on addition of heme.

Hemoglobin: 6.67×10^{-7} M. Added heme: I, 0 M; II, 6×10^{-6} M; III, 1×10^{-5} M; IV, 1.6×10^{-5} M; V, 2×10^{-5} M; VI, 2.4×10^{-5} M; VII, 3×10^{-5} M; VIII, 4×10^{-5} M; IX, 6×10^{-5} M.

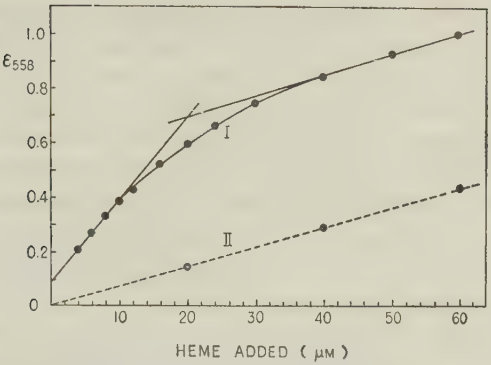


FIG. 7. Extinction at 558 mμ of alkaline denatured globin hemochrome as is increased on addition of heme.

I. Alkaline denatured globin hemochrome.

II. Control without protein.

composed of two linear portions with different slopes. The steeper slope corresponds to a concentration curve of alkaline hemochrome at 558 mμ and the milder slope is completely parallel with curve II, which is the concentration curve of heme at 558 mμ, which was determined by control of above noted reaction mixture, containing 1 ml. of water instead of oxyhemoglobin solution. The facts reveal that the rise in optical density in the steeper portion is due to the increase of globin-combined heme, whereas in the milder portion it is based on the augumentation of free heme, not

TABLE I

Combination of Heme to Proteins

	Horse hemoglobin	Bovine serum albumin	Horse serum albumin	Human serum γ-globulin
Concentration* of protein (μM)	0.67	0.97	1.94	2.2
Total heme combined (μM)	22.7	13	29	34
No. of combined heme per molecule	34	13	15	16
No. of heme linked groups per molecule	68	26	30	32

* Molecular weights of horse hemoglobin, horse serum albumin, bovine serum albumin, and human serum γ-globulin are referred to 68,000 (*11*), 70,000 (*7*), 70,000 (*12*), and 156,000 (*13*), respectively.

combined with globin. Therefore, the intercept of extrapolated two lines is assumed to correspond to the maximal amount of heme, combined to globin. The optical density of 0.08 is observed in curve I at 0 M of heme. This is due to the extinction of hemes originally contained in hemoglobin.

The maximal amout of total heme combined to denatured globin, is calculated to be 2.27×10^{-5} M from the following equation.

Total heme=Newly combined heme+Originally combined heme

The maximal number of total heme per molecule of hemoglobin is calculated to be 34. Therefore, the number of heme linked groups per molecule of denatured hemoglobin is $2\times34=68$.

The combination of heme to other proteins was investigated by the same procedure as described just above, except that the calculation of original heme was omitted. The results are summarized in Table I.

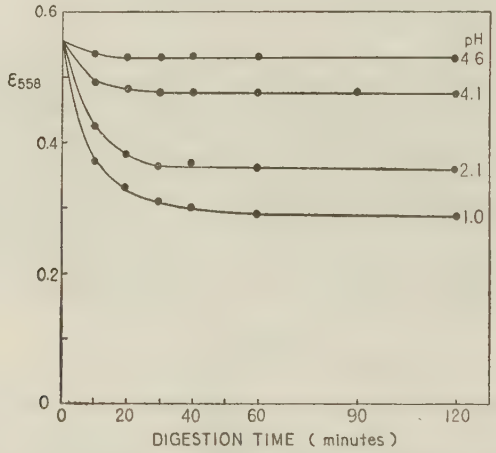


FIG. 8. Decrease of heme affinity of hemoglobin caused by pepsin digestion.

III. Combination of Heme with Proteinase Digested Proteins—Hemoglobin was digested by pepsin and the formation of alkaline hemochrome was estimated at 558 mμ as previously described. Fig. 8 shows the results obtained at various pHs. Optical density at 558 mμ decreased significantly with digestion time. The fact indicates that the affinity of globin to heme decreases markedly with the progress of protein hydrolysis and consequently the major parts of added heme remain in free state, not combined with the digests.

The percent combination of heme to digest was calculated from the following equation:

$$\text{Percent combination}=\frac{E'-E_0}{E_{100}-E_0}\times100$$

where E′ is the extinction measured, and E_0 and E_{100} are the extinctions of free heme and of 100 per cent combined alkaline hemochrome at 558 mμ,

respectively. In this experiment, the concentration of heme is 2×10^{-5} *M*. Hence, E_0 and E_{100} are 0.16 and 0.70. These values are obtained from the data of Fig. 7. Broken line in Fig. 9 (A) shows the relation of percent combination of heme *versus* digestion time, at pH 1.9. After 120 minutes of digestion, only 22 per cent of total heme is combined to the digest, while 74 per cent of total heme is combined to denatured globin, before digestion. Thus, the combination percent decreases by 52 per cent. Regarding the decrease of 52 per cent as 100 per cent, relative percent decrease of heme combination is replotted in Fig. 9.

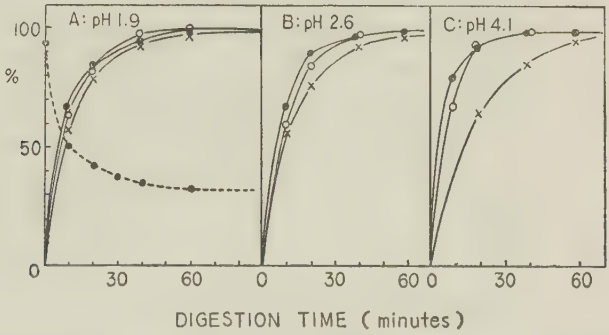


FIG. 9. Percent decrease of heme combination, and percent increases of color development by Folin's and biuret reagent, caused by pepsin hydrolysis.

●: percent decrease of heme combination. ○: percent increase of color development by Folin's reagent. ×: percent increase of color development by biuret reagent. Broken line: percent combination of heme.

The degree of digestion was also checked on the deproteinized filtrate as previously mentioned, by Folin's test and biuret test concomitantly in the same experiment. These results are also represented as relative percent increase of color development by each test and summarized in Fig. 9. From these figures it is recognized that the curve of percent decrease of heme combination is relatively well coincided with the curves of percent increase of color development by Folin's test and biuret test, in digestion at pH 1.9. With the rise of pH, however, the latter two test tend to delay, especially remarkably in the case of biuret test at pH 4.1. These facts imply that the affinity of heme decreases already in earlier stages of digestion, where the hydrolysis of globin proceeds only to some small extent. When the biuret test was carried out for the reaction mixture without trichloroacetic acid treatment, the rise in optical density at 540 mμ was almost negligible, as compared to that of the deproteinized filtrate. It is notable that the combination of copper ion to protein is little affected, whereas the combination of heme to protein decreases strikingly. Further digestion with trypsin was carried out as follows. Pepsin digestion of hemoglobin at pH 2.6 was stopped by addition of NaOH after 90 minutes and pH was raised to 9.5.

Hydrolysis was succeeded by addition of 2.5 mg. trypsin to this solution. Further decrease in optical density at 558 mμ was found to take place, concomitantaly with the rise in the optical density at 720 mμ, as shown in Fig. 10. At the end of pepsin hydrolysis, combination percent of heme to the digest was calculated to be 34 per cent. By further digestion with trypsin for 90 minutes this value decreased to 20 per cent (correction was made for dilution). From this fact it may be assumed that the polypeptides liberated from hemoglobin by pepsin are hydrolysed to the peptides of much lower molecule and consequently the affinity of the digests to heme decreases much more extensively.

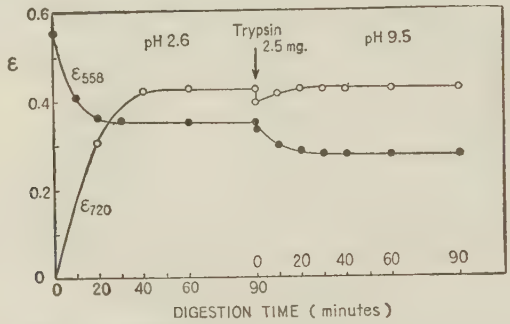


FIG. 10. Influences of trypsin hydrolysis carried out after pepsin hydrolysis, on heme affinity and color development by Folin's test.
●: extinction at 558 mμ of alkaline denatured globin hemochrome.
○: extinction at 720 mμ by Folin's test.

DISCUSSION

Alkaline denatured proteins are capable of combining with much of hemes, as may be seen from the data presented in Table I. The number of heme linked groups per molecule of denatured proteins is twice of the number of combined hemes, since the mode of heme combination in denatured proteins is, like that in the case of cytochrome c, of hemochrome type, which is known to be different from the mode of heme combination in native hemoglobin. The number of heme linked groups thus obtained are too much in excess, as compared with histidine contents in any cases, as listed in Table II. The fact seems to indicate that the heme linking groups other than histidine are involved in the combination. The fact that curve I of Fig. 7 showed a gentle bending, also may suggest the possibe participation of carboxyl group in the heme linkage; however, this possibility is excluded by the following fact. Fig. 11 shows that the increases of extinction at the isosbestic points (570.5 and 503 mμ) between alkaline denatured globin hemochrome and free heme, are straight lines when plotted against the quantity of added heme. If someother optical component is present in the system, giving different absorption spectrum from those of alkaline

TABLE II

Basic Amino Acids in Proteins and Their Combination Ratios in Heme Linkage

	Horse hemoglobin (*14*)	Bovine serum albumin (*12*)	Human serum γ-globulin (*13*)
Total amino acids	542	575	1305
Histidine	36	18	25
Arginine	14	24	43
Lysin	38	57	86
Tryptophan	5	2	22
NH_3-N	36	43	124
Total base (a)	129	144	300
No. of heme linked groups (b)	68	26	32
(b)/(a) × 100	53	19	11

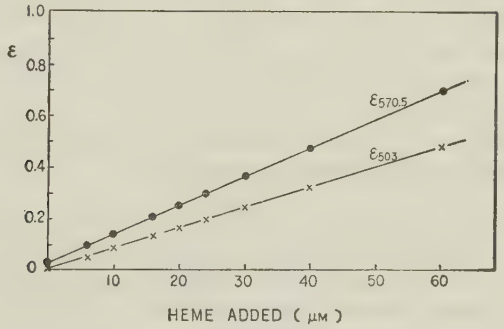


FIG. 11. Relation of the extinction at the isosbestic points (570.5 and 503 mμ) *versus* the quantity of added heme as measured in the optical system consisting of alkaline denatured globin hemochrome and heme.

hemochrome and free heme, increases of extinction at the isosbestic points will not follow a straight line. Hence, the bending of curve I in Fig. 7 appears to be caused by some basic amino acids other than histidine, which have weaker affinity than histidine, although they can form alkaline hemochrome like histidine. Table II summarizes the data showing the contents of these basic amino acids.

Combination ratios of these basic amino acids in proteins are remarkably higher than those of free amino acids. Especially in the case of denatured hemoglobin, it amounts to 53 per cent. In contrast, the combination ratio of free histidine to hematin is only 0.02 per cent approximately, even in

the best condition at pH 7.0. Similar value is semiquantitatively obtained in the combination of histidine to heme. Such a difference in the affinity seems to be attributable mainly to the difference of molecular size, namely the chain length of polypeptide. This is also supported by the fact that the digestion of protein to lower peptides resulted in a significant decrease of the affinity. The reason why polypeptidization of amino acids increases their affinity towards heme, remains obscure.

SUMMARY

1. Dissociation of histidine hemichrome coincided well with a theoretical second order dissociation curve. The change of pK in function of pH was elucidated by taking into consideration the dissociations of imidazole-NH of histidine and of hematin iron. The following theoretical equation was found to account for the results obtained.

$$pK = pK_0 - 2\log(1+[H^+]/K_a) - \log(1+K_b'/[H^+])$$

pK_0 was found to be 3.6 on an average, at room temperature of 20°.

2. Alkaline denatured proteins combined with much more heme molecules than expected from their histidine contents. The number of heme linked groups was estimated as 68, 26, 40 and 32 in horse hemoglobin, bovine serum albumin, horse serum albumin and human serum γ-globulin, respectively. The heme linked groups of alkaline denatured proteins were deduced to be not carboxyl groups of acidic amino acids but nitrogenous groups of basic amino acids.

3. The affinity of alkaline denatured protein towards heme was decreased remarkably by proteinase digestion.

The authors wish to express their gratitude to Miss M. Shinoda for her technical assistance, to Dr. H. Hirai for the gift of horse serum albumin and human serum γ-globulin, and to Dr. T. Kusunoki for the sample of bovine serum albumin. Thanks are also due to the Scientific Research Fund of the Ministry of Education for grant. The essential part of this study was published in Japanese elsewhere (*15*, *16*).

REFERENCES

(*1*) Holden, J., *Australian J. Exptl. Biol. Med. Sci.*, **16**, 153 (1938)
(*2*) Horiguchi, M., *J. Japan. Biochem. Soc.*, **25**, 218 (1953)
(*3*) Chu, T. C., and Chu, E. J., *J. Biol. Chem.*, **212**, 1 (1955)
(*4*) Willstätter, R., *Ann. Chem.*, **373**, 232 (1910)
(*5*) Suzuki, M., Kajita, A., and Hanaoka, C., *J. Biochem.*, **41**, 401 (1954)
(*6*) Cohn, E. J., Hughes, W. L. Jr., and Weare, J. H., *J. Am. Chem. Soc.*, **69**, 1753 (1947)
(*7*) Keckwick, R. A., *Biochem. J.*, **32**, 552 (1938)
(*8*) Dixon, M., *Biochem. J.*, **55**, 161 (1953)
(*9*) Greenstein, J. P., *J. Biol. Chem.*, **101**, 603 (1933)
(*10*) Shack, J., and Clark, W. M., *J. Biol. Chem.*, **171**, 143 (1947)
(*11*) Svedberg, T., and Fahraeus, R., *J. Am. Chem. Soc.*, **48**, 430 (1926)

(*12*) Velick, S. F., and Ronzoni, E., *J. Biol. Chem.*, **173**, 628 (1948)
(*13*) Brand, E., *Ann. New York Acad. Sci.*, **47**, 187 (1946)
(*14*) Tristram, G. R., The Proteins, edited by H. Neurath and K. Bailey, Academic Press Inc., New York. Vol. **I**, Part **A**, p. 181 (1953)
(*15*) Kajita, A., and Uchimura, F., *J. Japan. Biochem. Soc.*, **26**, 484 (1954)
(*16*) Mizutani, H., *J. Japan. Biochem. Soc.*, **27**, 545, 550 (1955)

The Journal of Biochemistry, Vol. 46, No. 5, 1959

STUDIES ON THE CHEMISTRY OF WAX D OF BCG

II. ON THE CHEMICAL STRUCTURE OF OLIGOMANNOINOSITIDES

By SHOSHICHI NOJIMA

(*From the Department of Chemistry, National Institute of Health, Tokyo*)

(Received for publication, September 16, 1958)

Phosphoinositides, first evidenced in human type tubercle bacilli by Anderson (*1*) in 1930, have been found in many animal and vegetable tissues such as brain and plant seeds. An excellent review concerned with their chemistry was recently presented by Folch (*2*).

Anderson and his collaborators abvanced the stuby of the tubercle bacilli phosphatides as one of their extensive works on lipides of acid-fast bacteria. Thus, Anderson, Lothrop and Creighton (*3*) have isolated a phosphorylated mannose inositol glycoside and glycerophosphate from the phosphatides of human type tubercle bacilli upon saponification in alkaline media. Further hydrolysis of this organic phosphate ester with dilute ammonia gave an amorphous oligosaccharide which had no reducing power and which was named as manninositose because mannose and inositol were liberated in a molecular ratio of approximately 2: 1 by the hydrolysis with sulfuric acid. Recently, phosphatide fractions which corresponded analytically to phosphatidylinositodimannoside were isolated by Lederer and his collaborators from the lipides of a streptomycin resistant strain of H37Rv (*4*) and of BCG (*5*). They have proposed a structural formula for the phosphatide on the basis of paper chromatographic identification of some hydrolysis products and the isolation of manninositose.

In a preceding paper, the author reported that inositol was found to be relatively abundant in Wax D as well as in a phosphatide fraction of BCG (*6*). Subsequently, phosphoinositide fractions were isolated from Wax D of BCG (*7*), suggesting a possibility that they might be new phosphoinositides containing oligomanno-ose which were tentatively designated as oligomannoinositides. However, there may be an alternative explanation; namely, that the phosphatide fraction from Wax D might be a mixture of phosphatidylinositodimannoside, as proposed by Lederer (*5*, *6*), and oligosaccharides of mannose. It is the main object of this paper to decide whether the phosphatides of Wax D are composed of oligomannoinosides or whether they are only a mixture of phosphatidylinositodimannoside and oligosaccharides. The difficulties encountered in obtaining pure lipide compounds emphasize the importance of making this differentiation (*2*, *8*). The present paper describes the isolation of pentamannoinositide (acylphosphatidylinositopentamannoside) in a relatively pure state. The barium salt of its

deacylated phosphodiester was also obtained in a crystalline state.

MATERIALS AND METHODS

Paper Electrophoresis—A sample of a phosphate ester which corresponds to 10-40 μg. P was applied to Whatman No. 1 which was then equilibrated about one hour with a 500 ml. aqueous solution containing 10 ml. of pyridine, 2 ml. of acetic acid and 30 ml. of *n*-butanol (pH 5.6)*. Further conditions are specified in the Table.

Infrared Spectra—were determined with a Hitachi infrared spectrophotometer Model EPI-2 using a rock salt prism. The samples were melted**.

Reversed-Phase Chromatography—The procedure used in this study, followed essentially the methods of Silk and Hahn (*9*). All other materials and methods described in the present paper were given in the preceding papers (*6*, *7*). The lot numbers of the phosphatides were shown in Table IV of the preceding paper (*7*).

RESULTS

Composition of One of the Phosphatides from Wax D of BCG—Table I shows that all the components of the phosphatides of Lot 3-b, which was a slightly

Table I

Composition of a Fraction of Phosphatides from Wax D of BCG; Balance.

Component	Weight distribution (*per cent*)	Molar distribution
Phosphoric acid	4.43	1.00
Glycerol	4.70	1.03
Inositol	6.40	0.72
Mannose	51.00	5.67
Fatty acid	42.2	2.99
Magnesium[1)]	0.54	
Total	109.27	
Correction[1)]	8.1	
Corrected total	101.17	

1) Magnesium was assumed to be present in an amount equivalent to in the phosphatide.

2) This correction allows for 10 molecules of water eliminated by bonding.

yellow colored powder, could be accounted for analytically. The approximate molecular ratio of glycerol, phosphorus, inositol, mannose and fatty acid (as

* This solvent mixture was shown by Dr. O. Hoshino, Faculty of Pharmaceutical Sciences, Tokyo University.

** The author is very grateful to Dr. H. Watanabe, Institute for Infections Diseases, Tokyo University, for carrying out measurement of the infrared spectra.

stearic acid) was 1 : 1 : 1 : 5–6 : 3. Further purification of the phosphatides was abandoned, because upon chromatography of 100 mg. of the material on a silicic acid column in the same manner as already described (7), no sharp peak was obtained.

Mild Alkaline Hydrolysis of Oligomannoinositides

Identification of the Phosphate Ester in the Hydrolysate—Three portions, 4.965 mg., 4.750 mg. and 5.240 mg., of Lot 1-c were each dissolved in 1 ml. of moist benzene. To each was added 0.4 ml. of 0.25 per cent methanolic KOH, and the solutions were then refluxed for 30 minutes, one hour and two hours, respectively. After cooling and removing the solvent *in vacuo* at room temperature, the residue was suspended in a small amount of ice-cold water, slightly acidified with 1 *N* HCl and repeatedly extracted with ester. Ethereal solutions were combined and reserved for later studies. Each of the water-soluble portions, after expelling the remaining ether *in vacuo*, was made up to 5 ml. with water. Aliquots of these solutions gave negative reactions when tested for inorganic phosphate.

The next experiment was performed with the phosphatides of Lot 3-a in the same manner as just described above except that acetic acid was used as a neutralizing agent instead of hydrochloric acid and the final aqueous solution was made up to 10 ml. These results are shown in Table II.

TABLE II

Alkaline Hydrolysis of a Phosphatide from Fraction B

(*mg.*)	Heating time (*minutes*)	Total mannose (*μg.*)	Mannose (*%*)	Total phosphorus (*μg.*)	Phosphorus (*%*)	Paper[1] electrophoresis
10.160	10	4730	46.6	153	1.51	One spot
11.430	30	5430	47.5	170	1.51	One spot
11.055	120	5030	45.6	165	1.50	One spot

1) Sprayed with Hanes-Isherwood reagent as well as ammoniacal silver nitrate.

These results show that all of mannose and organic phosphate were released from the phosphatides after hydrolysis for only ten minutes and moreover they were stable during hydrolysis for at least two hours. There are further indications to suggest that the mannose molecules were combining glycosidically with organic phosphate in the saponification aqueous media: specifically, the hydrolysed aqueous solution did not reduce the Benedict solution unless it was hydrolysed with hydrochloric acid beforehand. Also, upon paper electrophoresis, the location of the spot of this hydrolysate was the same whether it were detected by Hanes-Isherwood reagent or ammoniacal silver nitrate reagent or ammoniacal silver nitrate reagent.

Table III shows that the organic phosphate in this hydrolysed solution is different from glycerophosphate or methylglycerophosphate. The following experiments suggest the glycerol is not free but is in a bound form, presumably as a phosphate ester. After paper electrophoresis of the hydrolysed solution, the same dried paper was subjected to paper chromatography towards the vertical direction against the direction of electrophoresis with the following solvent mixtures, spraying with ammoniacal silver nitrate: (1) *n*-butanol-acetic acid-water (4 : 1 : 5), (2) isopropanol-5 *N* NH_4OH (2 : 1) in ascending systems.

However, no spot corresponding glycerol was obtained. On the other hand, 10 mg. of Lot 3-a was hydrolysed in the same manner as described above and water-soluble portion was made up to 10 ml. After lyophilization of 8 ml. of this solution the residue was dissolved in 2 ml. of 6 *N* HCl and heated in a sealed tube at 100° for 38 hours. The solution diluted with water was deionized by passing through Dowex-2 (OH^-), IR-120 (H^+) and again Dowex-2 (OH^-) and concentrated *in vacuo* to a very small amount. The paper chromatography of the solution gave spots with R_f values of (1): 0.37 and (2): 0.77, which were identical with those of glycerol.

TABLE III

Mobilities of Glycerophosphorylinositolpentamannoside (GPIPM) and Other Phosphate Esters in Paperelectrophoresis

1)	pH 5.6 at 17.9 v./cm. for 90 min.	
	Inorganic phosphate	1.0
	Glycerophosphate	0.95
	Diphenylphosphate	0.83
	GPIPM	0.66
2)	pH 5.6 at 19.3 v./cm. for 100 min.	
	Inorganic phosphate	1.0
	Methylglycerophosphate	0.91
	GPIPM	0.64

Isolation of Phosphodiester from the Hydrolysate of Lot 3-a Two hundred mg of the phosphatides was dissolved in 6 ml. of moist benzene and 1.5 ml. of 2 per cent methanolic NaOH (the final NaOH concentration was 0.1 *N*) and refluxed for 2 hours.

The water-soluble portion obtained, after treatment in the same manner as described above, was placed on a column of Dowex-2 (OH^-) of 200 400 mesh (9 × 175 mm.). After washing the column with 100 ml. of water, elution of the column with NaOH was carried out as follows: 100 ml. of 0.05 *N* NaOH, 165 ml. of 0.1 *N* NaOH. Three ml. fractions were collected. The 35–55th tubes (F_2) of eluates obtained by 0.1 *N* NaOH gave the first distinct peak by anthrone reaction. This peak amounted to 54.6 per cent recovery of the material as calculated from the starting phosphatides. The 94–107th tubes (F_3) of eluates obtained by 1 *N* NaOH also gave a distinct peak amounting to a recovery of 10.5 per cent of the material. The fraction F_2 was converted to the barium salt by passing the solution through IR-120 (H^+) and IRC-50 (Ba^{++}). Approximately 200 ml. of aqueous solution were concentrated to about 10 ml. by lyophilization. After removal of the white precipitate by centrifugation the supernatant and washings of the precipitate were combined and subjected again to lyophilization. The resulting white powder was trituated twice with anhydrous acetone and dried. This was again dissolved in about 2.5 ml. of water and the insoluble material was removed by filtration. To the clear filtrate, about three volumes of absolute alcohol was gradually added and the resulting solution was allowed to stand for a day in an ice-box. The supernatant obtained by centrifugation of the solution was allowed to stand an additional two weeks. At the end of this time, a syrupy precipitate was collected by centrifugation. The syrup was trituated with a small amount of absolute alcohol to give a white heavy powder, which was again dissolved in about 1.5 ml. of water and precipitated with three volumes of absolute alcohol after removing insoluble material by filtration. Thus, the series of procedures, dissolution, filtration and precipitation, was repeated

twice more. The white powder finally obtained was again dissolved in 1.0 ml. of water giving a transparent solution and precipitated with a large excess of absolute alcohol. Finally, 15 mg. of a hygroscopic white powder with a decomposition point at 226° was obtained (Table IV).

TABLE IV

Analytical Figures of Glycerophosyhorylinositoldimannoside

	C (%)	H (%)	P (%)	Mannose (%)
Calcd. for I:	31.94	4.96	5.50	31.9
II:	*34.71*	*5.23*	*4.26*	*49.6*
III:	36.49	5.41	3.49	60.8
Found :	*34.49*	*5.92*	*4.08*	*56.2*

I: glycerophosphorylinositolmannoside barium salt ($C_{15}H_{28}O_{16}PBa/2$)
II: glycerophorylinositoldimannoside barium salt ($C_{21}H_{38}O_{21}PBa/2$)
III: glycerophosphorylinositoltrimannoside barium salt ($C_{27}H_{48}O_{26}PBa/2$)

This compound gave only one spot on paper by electrophoresis under the same conditions described in Table III spraying with Hanes-Isherwood reagent as well as with ammoniacal silver nitrate. It also gave only one spot on paper chromatography in a solvent mixture of $CH_3OH : HCOOH : H_2O$ (80 : 15 : 5) with an R_f value of 0.52 spraying with Hanes-Isherwood reagent. The eluate F_3 was also purified *via* the barium salt in the same manner described above and gave a white powder giving two spots on paper by electrophoresis, one of which corresponded to glycerophosphate.

Isolation of Phosphodiester from the Hydrolysate : from Lot 3-b —A sample of 230 mg. of the phosphatides were saponified and treated in the same manner as described above for Lot 3-a. From the fractions eluted with 0.1 *N* NaOH (F_5), a distinct peak which accounted for 50 per cent of the starting material could be detected by the anthrone method. Further elution with 0.2 *N*, 1 *N* NaOH and 1 *N* NaOH plus 1 *N* NaCl furnished almost negligible amounts of anthrone positive material (Fig. 1). The eluate F_5 was passed through IR-120 (H^+) to remove sodium. An aliquot of the solution was subjected to electrometric titration giving no secondary phosphate dissociation over the pH range from 4 to 11. Another aliquot of this solution did not give any precipitate with neutral lead acetate solution but furnished a white heavy precipitate on addition of basic lead acetate. These results strongly suggested that the ester was a phosphodiester. The remaining free acid was converted to the barium salt by passing through IRC-50 (Ba^{++}). Lyophilization of about 150 ml. of aqueous solution gave 111 mg. of a slightly yellowish hygroscopic powder with a decomposition point of 220° (P=1.68 per cent). The powder was dissolved in 5 ml. of water, the insoluble material was filtered and the filtrate was again lyophilized. After trituation of this material with a small amount of acetone, 90 mg. of a white powder (P=1.37 per cent) was obtained. Then the powder was redissolved in 2 ml. of water, the insoluble material filtered, and anhydrous acetone was added to the filtrate until a precipitate began to form. After standing overnight, centrifugation and decantation of the supernatant, viscous syrup like water-glass deposited at the bottom and on the wall of the

tube and it turned to a white powder by trituation with 4 ml. of acetone. A series of successive procedures, dissolution of the powder in water, precipitation by acetone and trituation with acetone, was repeated three times more and then a white powder was

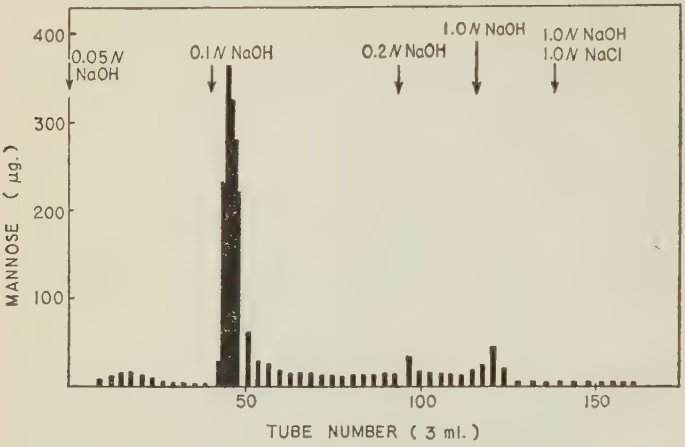


FIG. 1. Elution with NaOH of phosphate esters from pentamannoinositide absorbed on the colum of Dowex-2 (OH^-).

The column size was 9×175 mm, and 200–400 mesh of resin was used. The arrow indicates the position where the concentration of NaOH solution was changed. The quantity of mannose in 0.1 ml. of each tube was measured by anthrone reagent and expressed in μg. on the ordinate.

FIG. 2. Crystals of barium salt of glycerophosphorylinositolpentamannoside (×225).

obtained (P – 2.22 per cent). Further purification of this powder was repeated three times more with absolute alcohol instead of acetone in the same manner as described above. The resulting white powder was dissolved in 2 ml. of water yielding a transparent solution

without any insoluble material. Two ml. of absolute alcohol was added and the mixture was allowed to stand overnight. To the resulting syrup, was added a large amount of absolute alcohol for crystallization. Thirty mg. of crystals was obtained (Fig. 2). The crystals looked like very small sugar-candies, were very hygroscopic, softed at 225° and decomposed at 228°. To analyse the samples, it was necessary to heat them at 120° for

TABLE V

Analytical Figures of Glycerophosphorylinositolpentamannoside

	C (%)	H (%)	P (%)	Mannose (%)	Inositol (%)
Calcd. for I:	37.71	5.52	2.95	68.7	17.15
II:	*38.71*	*5.65*	*2.55*	*74.3*	*14.97*
III:	39.27	5.67	2.26	78.7	13.10
Found:	*38.37*	*5.55*	*2.56*	*73.3*	*15.01*

I: glycerophosphorylinositoltetramannoside barium salt ($C_{33}H_{58}O_{31}PBa/2$)
II: glycerophosphorylinositolpentamannoside barium salt ($C_{39}H_{68}O_{36}PBa/2$)
III: glycerophosphorylinositolhexamannoside barium salt ($C_{45}H_{78}O_{41}PBa/2$)

2 hours in *vacuo* (Table V). This compound did not reduce Benedict solution and gave only one spot on paper electrophoresis. Paper chromatography with a solvent mixture of $CH_3OH : HCOOH : H_2O$ (80 : 15 : 5) also gave only one spot with an R_f value of 0.40, when sprayed with Hanes-Isherwood reagent.

$$[\alpha]_D^{27}=+103° \text{ (c, 0.408 in } H_2O, \text{ l=1.0)}$$

In a similar experiment with a sample of 200 mg. of Lot 3-c, a better yield of the crystalline barium salt was obtained (59 mg.). Mannose=76.2 per cent.

Isolation of Phosphodiester from the Hydrolysate : from Lot 4—A sample of 230 mg. of the phosphatides furnished 25 mg. of the barium salt with decomposition point of 228°.

	P (per cent)	Mannose (per cent)
Calcd. for barium salt of glycerophosphorylinositoltetramannoside:	2.95	68.7
Found :	2.86	67.8

Re-chromatography of Glycerophosphorylinositolpentamannoside—A sample of 15.4 mg. of the crystalline barium salt from Lot 3-c was dissolved in a small amount of water, passed through IR-120 (H^+) and placed on a column of Dowex-2 (OH^-) of the same size as described above. The column was washed with 500 ml. of H_2O and then eluted with 100 ml. portion of NaOH solution whose normality varied from 0.06 *N* to 0.1 *N* in 0.005 *N* increments.

While fractions from 0.06 to 0.08 *N* alkali were anthrone negative, those obtained in elution with 0.085 *N* alkali were distinctly anthrone positive and accounted for 48 per cent of the starting material. Fractions A and B were collected as shown in Fig. 3. and found to have mannose/phosphorus ratios of 5.04 and 5.10, respectively.

Other Components

Hydrolysis of Phosphodiester—Mannose: Approximately 5 mg. of the free acid of the

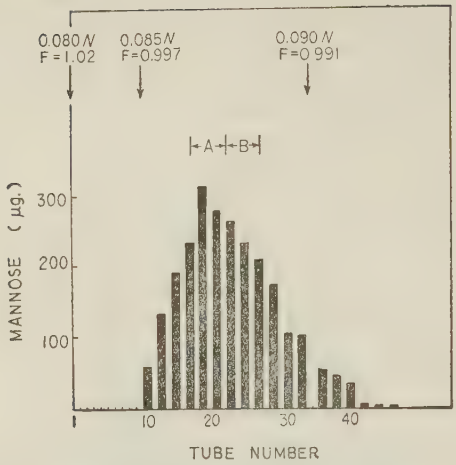


FIG. 3. Elution with NaOH of glycerophosphorylinositol-pentamannoside absorbed on the column of Dowex-2 (OH^-). The column size was 9×175 mm, and 200–400 mesh of resin was used. The arrow indicates position where the concentration of NaOH was changed. F is the factor of the normality of NaOH. Six-ml. fractions were collected. The amount of mannose in each fraction was expressed in μg. on the ordinate.

diester from Lot 3-b (glycerophosphorylinositolpentamannoside) was hydrolyzed with 3 *N* HCl for 2 hours in a boiling water bath. The cooled solution was passed over Dowex-2 (CO_3^{--}), and concentrated *in vacuo*. Paper chromatography of the residue was carried out with the following solvent mixtures spraying with aniline oxalate solution (1) 80 per cent aqueous phenol and (2) *n*-butanol-pyridine-water (6 : 4 : 3). In each case, the paper showed a single spot with R_f value identical to that of mannose; that is, 0.35 and 0.39 in the solvent systems (1) and (2), respectively.

Hydrolysis of Phosphodiester—Glycerophosphate and Inositolphosphate: Thirteen mg. of the barium salt of the diester from Lot 3-b (glycerophosphorylinositolpentamannoside) dissolved in a small amount of 6 *N* HCl was heated on a boiling water bath for two hours and dried *in vacuo*. The residue, dissolved in a small amount of water, was treated with IR-120 (H^+) to remove barium. The solution was applied to paper in a narrow streak about 6 cm. in length and chromatographed in a solvent mixture of $CH_3OHHCOOH$-H_2O (80 : 15 : 5). Two broad bands with R_f values of 0.29 (A) and 0.75 (B) were detected by spraying with Hanes-Isherwood reagent in the test development. Area (A) was cut off from the chromatogram and washed with 0.05 *N* NaOH to elute the phosphate ester. After passing the solution through IR-120 (H^+), it was made up to 6 *N* with conc. HCl and heated at 100° in a sealed tube for 40 hours. After taking the solution to dryness in a dessicator of solid NaOH dissolving again in water and passing through Dowex-2 (OH^+), the resulting solution was paperchromatographed with a solvent mixture of isopropanol-5 *N* NH_4OH (2 : 1) spraying with ammoniacal silver nitrate solution. An R_f value of 0.38 of the spot was identical with that of inositol (R_f of glycerol; 0.73 and R_f of mannose; 0.57). No other spot was detected. Therefore, (A) is a spot of inositolphosphate. In addition, (B) proved to be identical with the spot of glycerophosphate.

Fatty Acids from Lot 3-a—The combined ethereal solution obtained by extracting the saponification mixture of 200 mg. of the phosphatides was concentrated *in vacuo* under a stream of nitrogen. In order to hydrolyse methyl esters, a sample of 92 mg. of the dried residue was further refluxed with 10 ml. of alcoholic 1 *N* NaOH for 2 hours. The acids were isolated in the usual manner by acidification, extraction with ether and evaporation of the solvent. Yield: 78 mg. Melting point: 30–32°. Neutral value: 196. Iodine number: 0.

Fatty Acids from Lot 3-b—A sample of 230 mg. of the phosphatides gave 90 mg. of fatty acids melting at 28–32°. The neutral value: 196. Iodine number: 0. Fig. 4-A is an infrared spectrum of the acids showing a somewhat irregular bandprogression (*11*) between 1180 and 1350 cm.$^{-1}$ and a strong absorption band at 1380 cm.$^{-1}$, characteristic of a terminal methyl group. A sample of 36.720 mg. of this material was subjected to the reversed-phase column chromatography according to the method of Silk and Hahn (*9*). The results are shown in Fig. 5. The recovery of fatty acids by chromatography was 95 per cent of

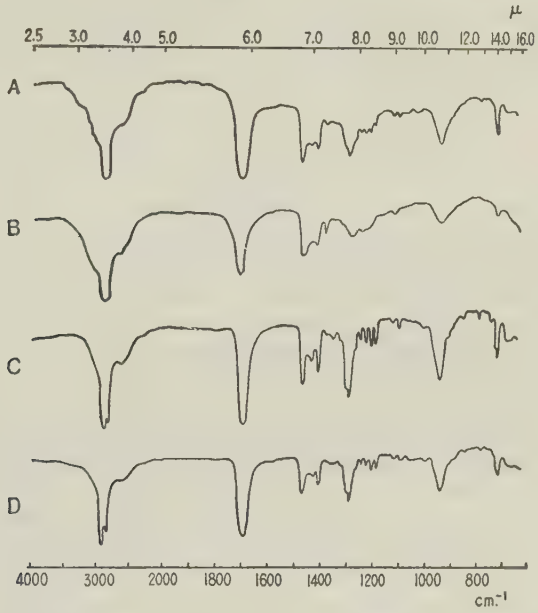


FIG. 4. Infrared spectra of fatty acids from pentamannoinositide (taken from melts).

A: Crude fatty acids from pentamannoinositide, B: An acid from A, C: m.p. 61–62° from A, D: An authentic sample of palmitic acid.

the starting material. Both the first fraction (C) in tubes 28 to 41 and the second (B) in tubes 50 to 65 were treated in the following manner. After concentration of the fraction it was acidified with 1 *N* HCl and repeatedly extracted with petroleum ether. The residue from the petroleum ether solution was converted to the sodium salt by 6 *N* NaOH. The resulting soap solution was repeatedly washed with ether and petroleum ether to extract liquid paraffin used in chromatography. The precipitate and aqueous solution were acidified with HCl and the lipide portion was obtained in the usual manner. (B) in Fig. 5: an acid having the neutral value of 188 (tuberculostearic acid (10-methyl-stearic acid); 188).

(C) in Fig. 5: m.p. 61–62°, neutral value; 213 (palmitic acid; 218). The acid (C) was identified as palmitic acid from its melting point, neutral value, infrared spectrum (Fig. 4-C) and a comparison of its chromatographic elution position with that of palmitic acid is shown in Fig. 5. As to the acids (B), a discussion will be given later.

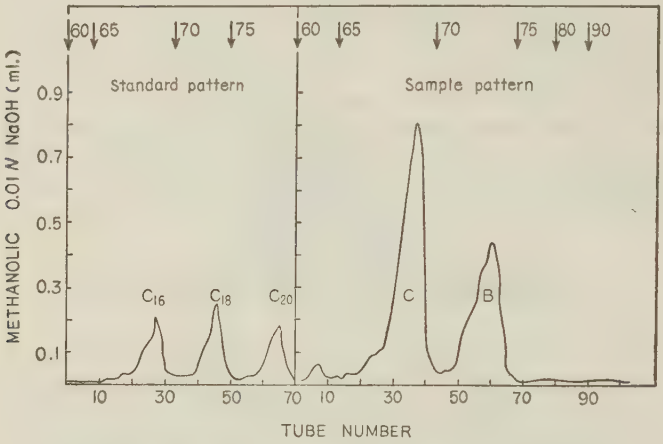


FIG. 5. Reversed-phase chromatograms of standard fatty acids and fatty acids from pentamannoinositide. A column (0.8×85 cm.), pecked with non-wetting kieselguhr impregnated with liquid paraffin, and the aqueous acetone-medicinal paraffin system were used. The column was maintained at 37° during the experiments. The arrow indicates the position where the concentration of aqueous acetone was changed. The number attached to each arrow shows the percentage of acetone in aqueous acetone. Development of the chromatography was begun with 40 per cent aqueous acetone. Then, 60, 65, 70, 80 and 90 per cent aqueous acetone previously equilibrated with paraffin were successively added to the column. The flow rate was from 15 ml. to 20 ml. per hour. Three-ml. fractions were collected and titrated with methanolic 0.01 *N* NaOH. Each tube was boiled before titration and bromthymol blue, as 0.1 per cent neutral solution in 70 per cent aqueous acetone, was used as an indicator. As a control, a mixture of 2.800 mg. of palmitic acid, 3.240 mg. of stearic acid and 2.695 mg. of arachidic acid, was placed on the column and the recoveries of the fatty acids from the column were 102, 102, and 112 per cent, respectively. A sample of 36.720 mg. of fatty acids from pentamannoinositide was placed on the column and 95 per cent of the material was recovered.

Metal—A sample of 3 mg. of the phosphatides from Lot 3-b was suspended in a small amount of water and dialysed against water in a cellophane bag for a day. The non-diffusable residue was made up to 1 *N* HCl aqueous solution and left standing for an hour with occasional shakings. The supernatant obtained by centrifugation was evaporated on a watch glass and the residue was again dissolved in several drops of water. One drop of this solution gave a strong positive reaction for magnesium according to the test with alkali hypoiodite described in Spot Test by Feigl (*12*). The remaining solution was applied to a paper and chromatographed with a mixture of methanol and concentrated HCl (10:3) spraying with 1 per cent oxine alcoholic solution. The air dried paper was exposed to NH_3 gas. Only one spot with an R_f value of 0.74, identical with that of magnesium, was

detected under ultraviolet light. No spot corresponding to calcium was detected*.

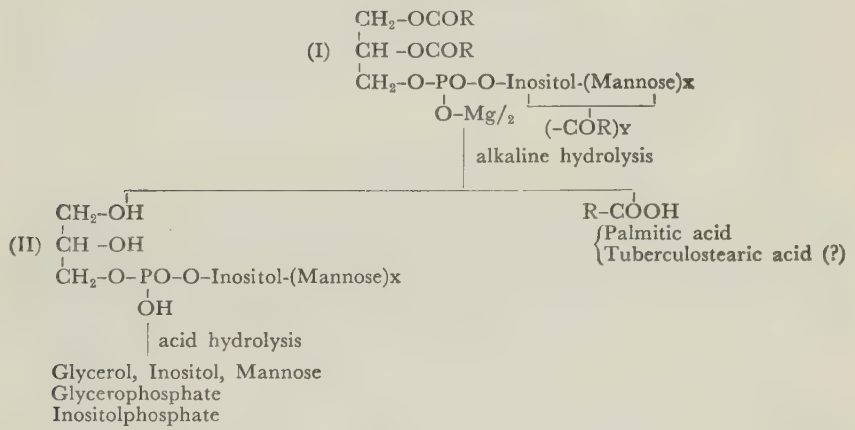
DISCUSSION

It was shown by Anderson (*13*) that the polysaccharides, isolated from the phosphatides of a strain of tubercle bacilli H37 grown in two separate cultures under the same conditions on Long synthetic medium, were distinctly different in their chemical compositions. Similarly, it was shown in the previous report of this series (*7*), that the phosphatides from Wax D of BCG grown under identical conditions on Sauton medium were distinctly different from one another in their content of mannose. The present study was undertaken to decide whether such differences in the chemical composition of the phosphatides from different cultures is due to the contaminants or to variations within the phosphatides molecules.

This work is presented in summary in the following two schemes (Scheme 1 and 2). The barium salt of glycerophosphorylinositolpentamannoside, which

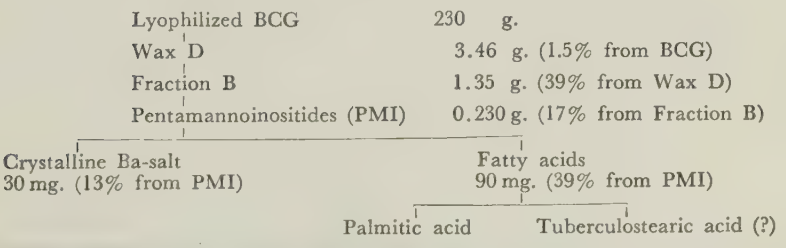
Scheme 1

Hydrolysis of Oligomannoinositides



Scheme 2

Isolation of Pentamannoinositide from BCG and Its Hydrolysis



* This chromatographic separation of metals was devised by Dr. Z. Tamura, Faculty of Pharmaceutical Sciences, University of Tokyo.

corresponds to (II) of Scheme 1 possessing an X value of 5, was obtained from Lot 3-b and -c in a crystalline condition and was proved to be homogeneous by re-chromatography. In contrast to these cases, a phosphodiester with two molecules of mannose was obtained from Lot 3-a. It may be identical with the phosphodiester already detected on paper chromatogram by Lederer (*4*, *5*). The diester isolated from Lot 4 involved four molecules of mannose. Although it was a powder, it gave only one spot by paper chromatography. The isolation of this phosphodiester suggests that the crude phosphatides of Lot 4 (mannose/phosphorus=18.3) were heavily contaminated with carbohydrate.

The general phosphodiester structure shown in (II) of Scheme 1 was further supported by identification of glycerophosphate and inositolphosphate among the acid hydrolysis products of the diester. However, at present, it is not possible to formulate with any certainty how the sugar moieties are attached in the phosphodiesters.

From the facts above described, oligomannoinositides can be represented by chemical structure (I) in Scheme 1. In the case of Lot 3-b, X is equal to 5 and Y to 1 (pentamannoinositides). In the case of Lot 3-a, X is equal to 2. No evidence for chemical combination between fatty acids and sugar was given in the present paper but such a structure as (I) in Scheme 1 may be probable because of the recent elucidation of the structure of cord factor in which 6 and 6' positions of trehalose were esterified by two molecules of mycolic acids (*14*).

As for the nature of the fatty acids, palmitic acid and a not yet identified acid were obtained. The infrared spectrum of the latter (Fig. 4-B) clearly indicated that it was not a long straight chain fatty acid because of the absence of a progression of bands of uniform intensity and spacing between 1180 and 1350 cm^{-1}. (*11*). The strong absorption of the C-CH_3 band at 1380 cm^{-1}. as compared with that of palmitic acid was considered to be a evidence for a branched chain fatty acid. The absence of the characteristic absorption band at 1020 cm^{-1}. indicated that this acid had no cyclopropane ring (*15*). Although the available data are not considered to be sufficient for a conclusive identification, the author would tentatively assume that the acid B (Figs. 4-b and 5) might be identical with tuberculostearic acid (10-methyl stearic acid) or its isomer with a branched chain (*16*). In this respect, it would be interesting to compare this acid with that prepared by Polgar (m.p. 23–24°) *via* acetol derivative from the neutral fat of tubercle bacilli (*17*), and with the acid of m.p. 10–11° prepared by Asselineau from H37 Ra without hydrogenation (*16*).

As shown in Table IV in the preceding report (*7*), a very small amount of arabinose was always detected by paper chromatography in addition to a large amount of mannose in the original phosphatides. Although the diester eluted with NaOH from the column still contained a trace of arabinose, the barium salt of the diester, purified by repeated precipitation, gave on spot of arabinose after hydrolysis. From these it may be concluded that the

oligomannoinositides were contaminated with a very small amount of phosphatides containing arabinose.

Since the isolation of phosphotides from tubercle bacilli by Anderson (*18*), the significance of the biological activities, especially of the serologic properties of this fraction, have been repeatedly emphasized by many investigators (*19*). However, the clear-cut demonstration of the relation between chemical structure of the phosphatides and their serological activities has not yet been established.

Recently, Pangborn (*20*) announced in a simple note the isolation of two serologically active saturated phospholipocarbohydrates from tubercle bacilli. The analytical figures of one of them (P 1.73, inositol 10.54, mannose 50 per cent; molecular ratio, phosphorus : inositol : mannose = 1 : 1 : 5) were very similar to those of pentamannoinositide described in the present study (Table I), but the lack of information concerning glycerol in Pangborn's description permits no decisive identification. It is of great interest that she found a close relationship between the phosphoinositides and a serological reaction.

Finally, the chemical structure of phytoglycolipide (PGL) from plant seed phosphatides, recently elucidated by Carter and his collaborators (*21*), is also of great interest. PGL contains a phosphoinositol-oligosaccharide moiety, the structure of which certainly suggests some possibilities for further characterization of the phosphoinosito1oligomannoside moiety in the phosphatides of the present study.

SUMMARY

The chemical structure of oligomannoinositides isolated from Wax D of BCG was proposed and discussed. Barium salts of glycerophosphorylinositoldi-, tetra- and penta-mannosides were isolated in pure states from oligomannoinositides. Fatty acids of oligomannoinositides were proved to be composed of palmitic acid and an acid having the neutral value of 188.

The author is very grateful to Dr. D. Mizuno, Chief of the Department of Chemistry, National Institute of Health,Tokyo, for his constant encouragement and kind guidance throughout experiments of this work. He also expresses his thanks to Prof. Dr. T. Ukita, of the Faculty of Pharmaceutical Sciences, University of Tokyo, for his constant encouragement and helpful suggestions during the course of this work. Thanks are also due to Miss E. Kondo of the Department of Chemistry, National Institute of Health, Tokyo, for her skillful technical assistance in every experiment of this work. Microanalyses for carbon and hydrogen were carried out by Miss S. Hara of the Department of Chemistry, National Institute of Health, Tokyo.

REFERENCES

(*1*) Anderson, R. J., *J. Amer. Chem. Soc.*, **52**, 1607 (1930)
(*2*) Folch, J., and LeBaron, F. N., *Canad. J. Biochem. Physiol.*, **34**, 305 (1956)
(*3*) Anderson, R. J., Lothroup, W. C., and Creighton, M. M., *J. Biol. Chem.*, **125**, 299 (1938)

(*4*) Vilka, E., and Lederer, E., *Bull. soc. chim. biol.*, **38**, 111 (1956)
(*5*) Vilka, E., *Compt. rend.*, **245**, 588 (1957)
(*6*) Nojima, S., Kondo, E., and Mizuno, D., *J. Biochem.*, **45**, 475 (1958)
(*7*) Nojima, S., *J. Biochem.*, **46**, 499 (1959)
(*8*) Hanahan, D. J., and Olley, J. N., *J. Biol. Chem.*, **231**, 813 (1958)
(*9*) Silk, M. H., and Hahn, H. H., *Biochem. J.*, **56**, 406 (1954)
Yamakawa, T., Matsumoto, M., and Suzuki, S., *J. Biochem.*, **43**, 63 (1956)
(*10*) Bandurski, R. S., and Axelrod, B., *J. Biol. Chem.*, **193**, 405 (1951)
(*11*) Jones, R. N., McKay, A. F., and Sinclair, R. G., *J. Amer. Chem. Soc.*, **74**, 2575 (1952)
(*12*) Feigl, F., Spot Test, 1., Inorganic Applications, Elsevier Publishing Company, Amsterdam, 4th ed., p. 209 (1954)
(*13*) Sütö-Nagy, G. I. de, and Anderson, R. J., *J. Biol. Chem.*, **171**, 749 (1947)
(*14*) Noll, H., Bloch, H., Asselineau, J., and Lederer, E., *Biohim. et Biophys. Acta*, **20**, 299 (1956)
(*15*) Hofman, K., and Lucas, R. A., *J. Amer. Chem. Soc.*, **72**, 4328 (1950)
(*16*) Asselineau, J., and Lederer, E., *Progr. Chem. Organ. Natural Products*, **10**, 180 (1953)
(*17*) Polgar, N., *Biochem. J.*, **42**, 206 (1948)
(*18*) Anderson, R. J., *J. Biol. Chem.*, **74**, 525, 537 (1927)
(*19*) Pangborn, M. C., *Farady Society Discussion*, **6**, 110 (1949)
(*20*) Pangborn, M. C., *Fed. Proc.*, **17**, 1016 (1958)
(*21*) Carter, H. E., Gigg, R. H., Law, J. H., Nakayama, T., and Weber, E., *J. Biol. Chem.*, **233**, 1309 (1958)

The Journal of Biochemistry, Vol. 46, No. 5, 1959

STUDIES ON THE METABOLIC FUNCTION OF BIOTIN

III. ACCUMULATION OF α-KETO ACIDS IN BIOTIN-DEFICIENT CULTURE OF PIRICULARIA ORYZAE*

By HIROHIKO KATSUKI

(From the Department of Chemistry, Faculty of Science, Kyoto University, Kyoto)

(Received for publication, September 17, 1958)

The oxidation of pyruvic and α-ketoglutaric acid has been reported by many workers (*1-5*) to be considerably retarded in biotin-deficient organisms. But the mechanism of the retardation has not been elucidated.

During the course of studies (*6*, *7*) on the metabolic function of biotin using the rice blast fungus *Piricularia oryzae* it was found that besides pyruvic and α-ketoglutaric acid a keto acid accumulated in biotin-deficient culture. As shown in a preliminary communication (*8*), the undetermined keto acid was isolated and proved to be dimethylpyruvic acid.

The present paper is concerned with the isolation of keto acids and the contents of coenzymes such as CoA, ATP and DPN**, which have a relation to α-keto acid oxidation, in the biotin-deficient mycelia. It is also concerned with a discussion on the mechanism of the retardation of α-keto acid oxidation.

EXPERIMENTALS

Cultivation of the Fungus—The fungus used was *Piricularia oryzae Cavara*, strain No. 1. The method of cultivation and the basal culture medium used were the same as reported in the previous papers (*6*, *7*) except the contents of biotin and thiamine, which were: 0.25 μg., 1000 μg. per liter in the biotin-deficient medium and 5.0 μg., 8.0 μg. per liter in the thiamine-deficient medium and also 5.0 μg., 1,000 μg. per liter in the normal medium respectively. In the biotin-deficient medium, unless otherwise indicated, 20 g. of calcium carbonate were added per liter to avoid acidification of medium.

Separation of α-Keto Acids—After thirteen days of cultivation in the biotin-deficient medium, the culture fluid was separated from mycelia and treated with 2,4-dinitrophenylhydrazine solution (saturated in 4 *N* HCl) until no more precipitation occurred, and then it was allowed to stand over night in an ice-box. The precipitated hydrazones were filtered, washed with dilute hydrochloric acid and water successively, and finally dried over sulphuric acid in vacuo. The dried hydrazones thus obtained were dissolved in acetone, filtered from a trace of protein contaminated and dried again in vacuo. Parts of hydrazones were dissolved in methyl alcohol containing 1 per cent of ammonia to convert them into their

* This work was supported by the Grant in Aid for Scientific Research from the Ministry of Education and a part of this work was announced at the Annual Meeting of the Japanese Biochemical Society held in Fukuoka, on October 31, 1956.

** CoA, coenzyme A; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide.

ammonium salts and dried *in vacuo*. The hydrazones thuso btained were dissolved in acetone and separated by chromatography. For the chromatographic separation "active alumina for chromatography" (150–200 mesh) prepared Wako Chemicals Ind. Co. and deactivated with methyl alcohol were used. The powder of alumina was suspended in acetone and poured into the tower (2.5×45 cm.). Soon after the alumina column was provided, the acetone solution of the ammonium salts of the hydrazones were poured into the column. For the first developing solvent, acetone was used until the neutral hydrazones moved downwards and passed through the column. In order to develop the hydrazones of keto acids, a mixed solvent consisting of acetone and methyl alcohol was used. The percentage of methyl alcohol in acetone was increased gradually corresponding to the developing of bands. When the developing rate was slow, methyl alcohol or even methyl alcohol containing 1 per cent of ammonia could be used. By these developing methods, 4 bands of the hydrazones of keto acids were obtained. Each band, thus obtained, was cut out and eluted with ammoniacal methyl alcohol. After evaporation of the solvent in vacuo, the ammonium salt of the hydrazone obtained was dissolved again in water and acidified with dilute hydrochloric acid. The precipitated free hydrazone was washed with water, dried *in vacuo*, and finally recrystallized from the solvent shown in Table I.

Analysis of the Coenzymes of the Mycelia—CoA (*9*), ATP (*10*), pyridine nucleotides (*11*) and unstable phosphate were analyzed with the mycelial extract. The extraction of the coenzymes was carried out either by heating the acetone powder of mycelia with water (100°, 5 minutes) or by homogenizing the fresh mycelia with cold 1 per cent trichloroacetic acid (0°, 3 minutes). The acetone powder of mycelia was prepared as follows: Fungal mycelia, freed from the culture medium, were washed with cold water three times and pressed between filter paper. Then they were torn in shreds and thrown into 30 volumes of cold acetone with stirring. After renewing the acetone three times, the dehydrated mycelia were filtered from acetone and dried in vacuo.

RESULTS

Accumulation of α-Keto Acids—In biotin-rich media the maximum growth of this fungus was reached at the 10th day of inoculation, and during the cultivation the pH of media (6.6) was found to be constant. On the other hand, in biotin-deficient media (without calcium carbonate) this fungus grew so slowly that it required 30 days to reach its maximum growth, and owing to the accumulation of acidic substances, the pH of medium decreased 6.6 to 4.6 during the first 13 days of cultivation. The biotin-deficient culture fluid after 13 days cultivation was separated from mycelia and treated with 2,4-dinitrophenylhydrazine. The hydrazones of keto acids thus obtained were separated chromatographically into 4 components (A_1, A_2, A_3 and A_4). The yield, melting point, and R_f value of each hydrazone are shown in Table I. A_1 and A_2 were identified as the hydrazone of α-ketoglutaric acid and the *trans*-isomer (*12*) of the hydrazone of pyruvic acid respectively. A_3 was shown to be the *cis*-isomer (*12–14*) of the hydrazone of pyruvic acid, and A_4, to be the hydrazone of dimethylpyruvic acid (*14*), from the results of the analysis shown below and also of their failures to depress the melting points of the authentic samples respectively*.

* The author is grateful to Dr. K. Imai, Kyoto University, for supplying the authentic sample of dimethylpyruvic acid.

Table I

Yields, Melting Points and R_f Values of the 2,4-Dinitrophenylhydrazones of α-Keto Acids Accumulated in the Biotin-Deficient Culture

2,4-Dinitrophenylhydrazone	A_1	A_2	A_3	A_4	Neutral hydrazones
Yield per 1 liter of culture media (*mg.*)	82	232	93	830	175
Melting point (Solvent used for recrystallization)	219° (Acetone-benzene)	217° (Acetone-acetic acid)	211° (Acetone-benzene)	188° (50% Ethyl alcohol) 198° (Benzene)	
R_f[1]	0.02	0.13	0.25	0.68	

1) Solvent system; the mixture of *n*-butanol and petroleum ether (4 : 1), saturated with water. Sample; dissolved in 0.1 *N* Na_2CO_3.

Analysis

A_3; Calcd. for $C_9H_8O_6N_4$: C 40.31, H 3.01, N 20.89
Found : C 40.53, H 2.97, N 21.06
A_4; Calcd. for $C_{11}H_{12}O_6N_4$: C 44.59, H 4.08, N 18.92
Found : C 44.90, H 4.03, N 18.95

The R_f value of each hydrazone also corresponded to that of the respective hydrazone of keto acids. The relative molar concentration of α-ketoglutaric, pyruvic and dimethylpyruvic acid accumulated were 0.25, 1.2 and 2.8, respectively.

When the mycelial felts previously grown in the biotin-rich culture medium were floated on the new biotin-deficient medium which was adjusted pH to 4.6 with phosphoric acid, accumulation of keto acids was not observed. And even when cultivated in the biotin-deficient medium containing calcium carbonate or sodium bicarbonate not to become acidic, large amounts of keto acids were found to be accumulated. These facts suggest that the accumulation of keto-acids is not attributed to the acidity of the medium.

Contrary to what was expected, in the thiamine-deficient culture, an accumulation of dimethylpyruvic acid could not be found, while large amounts of the hydrazones of pyruvic and α-ketoglutaric acid were isolated.

Effect of Biotin on the Biosynthesis of CoA—Dimethylpyruvic acid has been proposed to be a precursor of pantothenic acid (*15*). And an observation (*16*, *17*) concerning the existence of the close relationship between biotin and pantothenic acid was reported. The results of dimethylpyruvic acid accumulation in the biotin-deficient culture seemed apparently to give a support to a possibility, as to the metabolic function of biotin, of catalyzing a step of the biosynthesis of pantothenic acid—*e.g.* hydroxymethylation of dimethylpyruvic acid—directly or indirectly. To check this possibility, the biotin-replacing activities of the following compounds in the growth-promoting action were examined: pantothenic acid, folic acid, *p*-aminobenzoic

acid, choline, serine, glycine, methionine, valine and formic acid. But none of them or of their mixtures were found to be effective.

As it has been well known that CoA is a coenzyme of α-keto acid oxidation enzyme system, the decrease of CoA in the mycelia should be expected to cause keto acid accumulation. Table II shows the CoA content of the mycelia. Contrary to the expectation, no difference was found between the biotin-deficient mycelia and the biotin-rich mycelia.

Table II

CoA Content of the Mycelia

Exp. No.	Sample	CoA/g. of dry mycelia (μg.)
1	Biotin rich	15.6
	Biotin deficient	20.2
2	Biotin rich	12.2
	Biotin deficient	12.4

Effect of Biotin on the ATP- and DNP- Contents of the Mycelia—When the CoA assay experiment was carried out by means of Kaplan and Lipmann's method, the extract of the biotin-deficient mycelia was found to lack the activity of acetylating sulfanilamide without addition of ATP to the assay system. The extract of biotin-rich mycelia, however, showed powerful activity of acetylation without ATP as shown in Table III.

Table III

Activity of Sulfanilamide-Acetylation owing to ATP in Mycelia

Assay system[1]		Absorbance at 545 mμ owing to unchanged sulfanilamide	Acetylated sulfanilamide owing to ATP in sample (μg.)
Biotin rich	complete	0.071	
	complete, minus ATP	0.145	2.66
Biotin deficient	complete	0.052	
	complete, minus ATP	0.208	trace
Complete, minus sample		0.148	
Complete, minus sample, minus ATP		0.207	

1) The complete system contained the following in a final concentration (per liter): sulfanilamide, 0.08 mM; ATP 3.67 mM; sodium acetate, 26.7 mM; sodium citrate, 16.0 mM; cysteine hydrochloride, 3.33 mM; sodium bicarbonate, 53.4 mM and the extract of mycelia. Total volume, 1.50 ml. The reaction mixture was incubated for 2 hours at 37°.

These results suggest that considerable amounts of ATP should be contained in the biotin-rich mycelia while only small amounts in the biotin-

deficient one. This was proved to be so by the determination of the content of ATP in both extracts by means of myosin-myokinase system (*10*). The results are summarized in Table IV.

TABLE IV
ATP Content of the Mycelia

Sample	Extracting solvent	ATP per g. of dry mycelia ($\mu g.$)
Biotin rich, acetone powder	Hot water	4050
Biotin rich, fresh	Cold trichloro-acetic acid	5220
Biotin deficient, acetone powder	Hot water	trace
Biotin deficient, fresh	Cold trichloro-acetic acid	<540

TABLE V
Unstable P Content of the Mycelia

Sample	P per g. of dry mycelia	
	Inorganic ($\mu g.$)	Unstable ($\mu g.$)
Biotin rich	2280	3020
Biotin deficient[1)]	1610	1060
Biotin deficient	1840	765

1) Cultivated without calcium carbonate.

TABLE VI
Pyridine Nucleotides Content of the Mycelia

Sample	Pyridine nucleotides (as DPN) per g. of dry mycelia ($\mu g.$)
Biotin rich	375
Biotin deficient[1)]	trace
Biotin deficient	159

1) Cultivated without calcium carbonate.

This diminution in the amount of ATP should result in a depression of phosphorylation activity in the biotin-deficient mycelia. As was expected, the content of unstable phosphate compounds in the biotin-deficient mycelia was observed to be far less than that in the biotin-rich one as shown in Table V.

Pyridine nucleotides, which could be, for the most part, regarded as DPN, were also found to decrease in the biotin-deficient mycelia as shown in Table VI.

DISCUSSION

In the present study, the accumulation of dimethylpyruvic acid in addition to pyruvic and α-ketoglutaric acid was observed in the biotin-deficient culture of *Piricularia oryzae*. Dimethylpyruvic acid has been reported to be accumulated by some organisms, especially by fungi (*18–20*), under different conditions. At present, this acid is presumed to be formed from pyruvic acid in organisms (*21*) and to be subjected to oxidative decarboxylation (*22*) like other α-keto acids. In the thiamine-deficient culture of *Piricularia oryzae*, dimethylpyruvic acid also, like pyruvic and α-ketoglutaric acid, has been expected to be accumulated. But this was not the case. This problem remains to be explained, but the biosynthesis of dimethylpyruvic acid from pyruvic acid is probably inhibited in the thiamine-deficient mycelia.

The activity of the oxidation of α-keto acids is controlled by many factors. The decrease of a coenzyme or coenzymes of keto acid oxidation system, as one factor, may bring about the retardation of the activity. The remarkable diminution observed in the contents of ATP and also pyridine nucleotides in the biotin-deficient mycelia of *Piricularia oryzae*, suggests that biotin might have some relation to the formation of ATP and pyridine nucleotides directly or indirectly. The clarification of this problem also should be expected from further study.

SUMMARY

1. *Piricularia oryzae* accumulates pyruvic, α-ketoglutaric and dimethylpyruvic acid in the biotin-deficient culture. In the thiamine-deficient culture, it accumulates pyruvic and α-ketoglutaric acid, but does not accumulate dimethylpyruvic acid.

2. Biotin-deficiency scarcely has effect upon the biosynthesis of CoA from dimethylpyruvic acid in this organism.

3. The contents of ATP and pyridine nucleotides were found to be remarkably decreased in the biotin-deficient mycelia. Correspondingly the amount of unstable phosphate compounds was also observed to decrease under this condition.

The author is indebted to Prof. Shozo Tanaka, Kyoto University, for his helpful criticism and encouragement during the course of this work.

REFERENCES

(*1*) Pilgrim, F. J., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 237 (1942)
(*2*) Pilgrim, F. J., and Elvehjem, C. A., *J. Biol. Chem.*, **156**, 257 (1944)
(*3*) Summerson, W. H., Lee, J. M., and Partridge C. W. H., *Science*, **100**, 250 (1944)
(*4*) Shive, W., and Rogers, L. L., *J. Biol. Chem.*, **169**, 453 (1947)
(*5*) Cantino, E. C., *Trans. New York Acad. Sci.*, **15**, 159 (1953)
(*6*) Katsuki, H., *J. Chem. Soc.* (*Japan*), **76**, 941 (1955)
(*7*) Katsuki, H., *J. Chem. Soc.* (*Japan*), **77**, 482 (1956)

(*8*) Katsuki, H., *J. Am. Chem. Soc.*, **77**, 4686 (1955)
(*9*) Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **174**, 37 (1948)
(*10*) Bowen, W. J., and Kerwin, T. D., *J. Biol. Chem.*, **220**, 9 (1956)
(*11*) Le Page, G. A., *J. Biol. Chem.*, **168**, 623 (1947)
(*12*) Moriwaki, T., Katsuki, H., and Tanaka, S., *J. Chem. Soc.* (*Japan*), **76**, 1367 (1955)
(*13*) Katsuki, H., Sumizu, K., Moriwaki, T., Tanaka, S., and Hayashi, I., *Nature*, **181**, 639 (1958)
(*14*) Katsuki, H., Sumizu, K., Moriwaki, T., and Tanaka, S., *Bull. Chem. Soc.* (*Japan*), **31**, 665 (1958)
(*15*) Maas, W. K., and Vogel, H. J., *J. Bacteriol.*, **65**, 388 (1953)
(*16*) Ryan, F. J., Kunin, C., Ballentine, R., and Maas, W. K., *J. Bacteriol.*, **65**, 434 (1953)
(*17*) Purko, M., Nelson, W. O., and Wood, W. A., *J. Bacteriol.*, **66**, 561 (1953)
(*18*) Hida, T., *J. Shanghai Sci. Inst.*, Section IV, **1**, 201 (1935)
(*19*) Katagiri, H., and Tachibana, S., *J. Vitaminology* (*Japan*), **7**, 86 (1954)
(*20*) Ramachandran, K., and Walker, T. K., *Arch. Biochem. Biophys.*, **31**, 224 (1951)
(*21*) Adelberg, E. A., A Symposium on Amino Acid Metabolism, The Johns Hopkins Press, Baltimore, p. 419 (1955)
(*22*) Kinnory, D. S., Takeda, Y., and Greenberg, D. M., *J. Biol. Chem.*, **212**, 385 (1955)

The Journal of Biochemistry, Vol. 46, No. 5. 1959

STUDIES ON THE ALGAL CYTOCHROME OF C-TYPE

By SAKAE KATOH

(*From the Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo*)

(Received for publication, September 22, 1958)

The distribution of cytochromes in algae was first described as early as 1935 by Yakushiji (*1*), who also succeeded in extracting a cytochrome c from various sea weeds. Since then there have been scattered reports on algal cytochromes (*2–5*) which, however, have not been as systematically studied for their physical and chemical properties as were various cytochromes from other sources. Quite recently, a new form of c-type cytochrome was isolated by Nishimura (*6*) in this laboratory from a green flagellate, *Euglena gracilis var. bacillaris*. The remarkably high level of oxidation-reduction potential characterizing the new cytochrome was reminiscent of another representative of the group, *i.e.*, cytochrome f, which has been discovered in the chloroplasts of higher green plants. In the present series of studies aiming at the elucidation of cytochrome constitution of algal cells, a type of cytochrome with characteristics almost identical with that of the *Euglena* cytochrome has been found in various species of algae covering the families of Rhodophyceae, Phaeophyceae, Chlorophyceae and Cyanophyceae. In the following, the methods of isolation and purification, as well as the properties of this algal cytochrome will be briefly described.

MATERIALS, METHODS AND RESULTS

Porphyra tenera and *Monostroma nitidum* were obtained from the so-called "Nori-fields" around Tokyo and Ise Bays, Japan, respectively, where the sea weeds are cultivated for food. Other species of algae were collected by the author from several locations on the coast of Tokyo Bay. *Tolypothrix tenuis* grown under laboratory conditions was kindly provided from the Institute of Applied Microbiology, University of Tokyo.

Usually, the cytochrome is readily extracted by autolysing the algae in water, as previously described by Yakushiji (*1*); washed thalli were soaked in a sufficient amount of pure water, kept at 15–20° for 24–48 hours and strained through cloth. Disruption of algal cells by grinding with quartz sand in a mortar, followed by extraction with pure water or phosphate buffer (0.1 *M*, pH 7.0) was also useful. In the case of *Tolypothrix tenuis* the cells were suspended in water and broken by sonic oscillation at 10 kc. for 20 minutes.

The autolysate or homogenate prepared as described above was first centrifuged at $3{,}000 \times g$ for 10 minutes. To 100 ml. of the supernatant, 45 g. of ammonium sulfate was added with stirring and the precipitate formed was removed by centrifugation. Twenty-five grams of ammonium sulfate were added and the precipitate formed was collected by centrifugation and dissolved in 0.05 M K_2HPO_4. The fractionation with ammonium sulfate was repeated once more (or several times, if necessary), and the finally obtained precipitate was dissolved in water. With most of the red and blue-green algae, the product at this stage of purification was almost free from any contamination of phycobilins. In some algae, however, a considerable contamination of certain viscous substances often protracted the process of purification. In these circumstances, the cytochrome was further purified by the positive adsorption technique, using calcium phosphate gel as the adsorbent and a series of phosphate buffers of different pH as the eluant. In the case of *Monostroma nitidum*, the crude autolyse contained a bulk of b-type cytochrome besides the c-type one under investigation. The two components, however, were readily separated by treatment with calcium phosphate gel. More detailed accounts of the purification procedure will be reported in a subsequent paper to be published elsewhere.

TABLE I

Spectral and Oxidoreductive Characteristics of C-type Algal Cytochromes

Algae	Wave length of absorption Maxima (mμ)			Normal potential ($V.$)
	α-band	β-band	Soret-band	
Rhodophyceae				
Porphyra tenera	553	521	416	0.34
Grateloupia sp.	552	520	415	0.30
Gelidium amansii	553	521	416	—
Phaeophyceae				
Undaria pinnatifida	553	521	415	0.34
Chlorophyceae				
Ulva sp.	552	521	415	0.30
Monostroma nitidum	552	521	416	0.31
Cyanophyceae				
Tolypothrix tenuis	553	521	416	0.30

The spectral characteristics of the cytochrome are presented in Table I. All the samples thus far examined of the cytochrome showed a remarkable coincidence with respect to the positions of absorption maxima in the reduced form; α-band: 552–3 mμ, β-band: 520–1 mμ and the Soret-band: 415–6 mμ. In the oxidized form, there appeared a broad band at 530 mμ and a Soret-band at about 408 mμ (see Fig. 1). The isolated cytochrome was found to be homogeneous with respect to the electrophoretic and column chromatographic

patterns*, thus excluding the possibility that the above-described spectrum of the cytochrome might represent a mixed bands of cytochromes c and f (*cf.* (*2*)). Alkaline pyridine and cyanide hemochromogens were prepared as described by Vernon and Kamen (*7*). The absorption spectra of the hemochromogens obtained were identical with that obtained in the case of mammalian cytochrome, indicating the identity of the porphyrin involved in both cases.

The measurement of the oxidation-reduction potential was performed by the spectrophotometric method with ferri- and ferrocyanide as the redox buffer. The results obtained are presented in the last column of Table I. The values for the normal oxidation-reduction potential E_0' at 20° and pH 7.0 ranged between 0.30–0.34 v.**. Therefore, the cytochrome under investigation belongs to the group of so-called high-potential cytochromes of c-type, which are known to be distributed in green leaves of higher plants, and in the cells of photosynthetic bacteria (*7*). It should be noted that the *Euglena* cytochrome isolated by Nishimura shows an oxidation reduction potential as high as 0.36 v. (pH 7.0, 25°).

Noteworthy is the fact that the c-type cytochromes obtained from widely different algal forms were practically identical in respect to their normal potentials and absorption spectra. The *Euglena* cytochrome may also be classed into this group of hemoproteins. It seems relevant to designate this group of cytochromes as "algal cytochrome 552–3; +300–360 mv."; the addition of the last term may be pertinent because there have already been discovered several different forms of cytochrome which show the main absorption peak at 552–3 mμ, but with significantly lower normal potentials.

The ubiquitous occurrence of this cytochrome among various algal forms seems almost indubitable. In other species not listed in Table I, *e.g. Vaucheria Nicorcii*, (a fresh water alga) and *Monostroma latissima*, the occurrence of the cytochrome was also indicated, as judged by the appearance of the absorption at 552–3 mμ in crude extracts. Lundegardh (*4*) as well as Duysens (*3*, *5*) have reported that the algal cytochrome in *Porphyridium* and *Chlorella* cells undergoes a photooxidation on illumination. Although the data for the oxidation reduction potential of the cytochrome involved are lacking in their reports, the similarity in the spectral characteristics indicates most plausibly the identity of their cytochrome with that described in this paper.

Further investigations on the biochemical properties and physiological functions of this algal cytochrome are in progress.

---

* unpublished data to be reported elsewhere.

** In an earlier paper, Yakushiji and Okunuki (*8*) mentioned on the occurrence of cytochrome c_1 in *Porphyra tenera*. The substance is most plausibly identical with that investigated in the present study, since the methods of preparation adopted were quite the same in both cases. The oxidation-reduction potential of the substance, however, evidently indicates that the component in question was not to be classed under c_1-group.

It is a pleasure to acknowledge the continuous interest and advice of Prof. H. Tamiya during the experiment. The author also wishes to thank Prof. A. Takamiya for his encouragement and valuable suggestions throughout this work.

REFERENCES

(*1*) Yakushiji, E., *Acta Phytochimica*, **8**, 325 (1935)
(*2*) Davenport, H. E., and Hill, R., *Proc. Roy. Soc.* (*London*) **B, 139**, 327 (1954)
(*3*) Duysens, L. N. M., *Science*, **120**, 353, (1954)
(*4*) Lundegardh, H., *Physiol. Plantarum*, **7**, 375 (1954)
(*5*) Duysens, L N. M., *Science*, **121**, 210 (1955)
(*6*) Nishimura, M., *J. Biochem.*, **46**, 219 (1959)
(*7*) Vernon, L. P., and Kamen, M. D., *J. Biol. Chem.*, **211**, 640 (1954)
(*8*) Okunuki, K., and Yakushiji, E., *Proc. Imp. Acad.*, **17**, 263 (1941)

The Journal of Biochemistry, Vol. 46, No. 5, 1959

CRYSTALLINE PHYCOBILIN CHROMOPROTEIDS OBTAINED FROM A BLUE-GREEN ALGA, TOLYPOTHRIX TENUIS

By AKIHIKO HATTORI and YOSHIHIKO FUJITA

(*From the Institute of Applied Microbiology, University of Tokyo, Tokyo*)

(Received for publication, September 25, 1958)

There is an extensive literature dealing with the physical and chemical nature of phycobilin pigments —phycocyanin, phycoerythrin and allophycocyanin— which represent, besides chlorophylls and carotenoids, important colored constituents of red and blue-green algae. From various red algae, which had mostly been used as materials in earlier studies of these pigments, phycoerythrin and phycocyanin have been isolated in crystalline state (*1–3*). Using a blue-green alga, *Aphanizomenon flos aquae*, as a material, Svedberg and Katsurai (*4*) obtained phycocyanin, but not phycoerythrin, in crystalline form. In contrast to these substances, allophycocyanin has never been isolated in pure form, and consequently little has been known about its physicochemical properties. The data reported on the physicochemical nature of the crystalline preparations of phycoerythrin and phycocyanin also seem to require reconsideration, since the sample studied by earlier workers were those obtained by simple repetition of salting-out procedures and, therefore, could not fully claim their chemical purity. Recently, Haxo *et al.* (*5*) applied for separating the pigments the chromatographic technique using tricalcium phosphate-gel as originally attempted by Swingel and Tiselius (*6*). By this method Haxo *et al.* succeeded in separating the three pigments in a clear-cut manner, although they did not proceed to the crystllization of each component.

The present work is concerned with the isolation and crystallization of the three phycobilin pigments by combined use of salting-out method and adsorption method. The algal material used was *Tolypothrix tenuis*, a fresh water bluegreen alga having the capacity of nitrogen fixation. Using the purified samples, the physical and chemical properties of each pigment were investigated in detail, and based on the results obtained, a critical appraisal was made of the reported by earlier workers.

EXPERIMENTAL

Algal Culture—Tolypothrix tenuis was grown as described in a previous paper (*7*) using a culture medium of the following composition: KNO_3, 3.0 g.; $MgSO_4 7H_2O$, 0.5 g.;

* This work was presented at the Annual Meeting of the Japanese Biochemical Society held in Sapporo on July 14, 1958.

$Na_2HPO_4 12H_2O$, 0.5 g.; $FeSO_4$, 0.04 g.; $CaCl_2$, 0.002 g.; Arnon's A_5 solution, 1 ml. per liter. The culture was constantly aerated with air containing 5 per cent CO_2 under illumination with incandescent or fluorescent lamps. Culture temperature was 32°. The cells grown under incandescent light were blue-green in color, while those grown under fluorescent light appeared dark brown, and it was found that phycocyanin predominated in the former and phycoerythrin in the latter. These forms, which may be designated in the following as "C-cells" and "E-cells", respectively, were conveniently used for obtaining different pigment components. After 2 or 3 weeks of incubation, the cells were collected by centrifugation, washed 2 or 3 times with pure water and used for the subsequent experiments.

Isolation of Phycobilin Pigments—Phycocyanin and Allophycocyanin from "C-cells": Thirty grams (dry weight) of "C-cells" were suspended in about 300 ml. of distilled water and thoroughly disintegrated by sonic oscillation at 10 kc for 20 minutes. The treated suspension was centrifuged at about $20{,}000 \times g$ for 15 minutes to remove cell fragments. The supernatant solution was bluish green in appearance, being contaminated with particles carrying chlorophyll and carotenoids. These were removed by centrifugation at $100{,}000 \times g$ for 60 minutes. The clear supernatant solution contained only phycobilin pigments (in

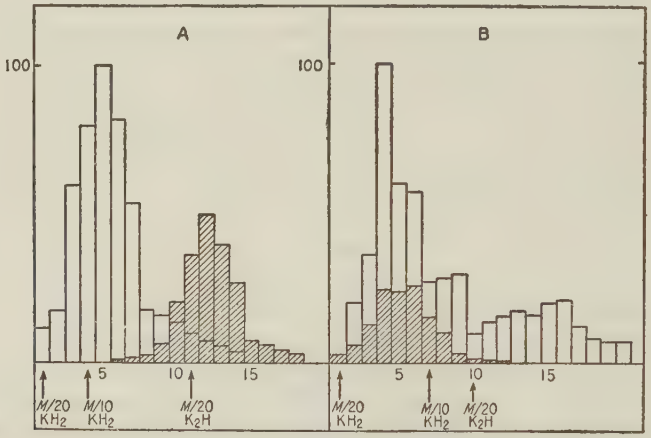


FIG. 1. Fractionating elution of phycobilin pigments which had been adsorbed on tricalcium phosphate-gel at neutral pH. The gel was successively treated with potassium phosphate solutions (20 ml. each) as indicated, and the contents of pigments were measured spectrophotometrically with the elutes separated from the gel by centrifugation. Ordinate: relative amounts of pigments in each elute as expressed in percentage of the highest level reached; abscissa: sequence numbers of elution. A, separation of phycocyanin and allophycocyanin; white bar: phycocyanin, shaded bar: allophycocyanin. B, separation of phycoerythrin and phycocyanin; white bar: phycoerythrin, shaded bar: phycocyanin.

this case, phycocyanin and allophycocyanin). On adding to the supernatant ammonium sulfate up to 50 per cent saturation, the bulk of phycocyanin was precipitated (P-I), leaving allophycocyanin in the solution. When the ammonium sulfate concentration was increased to 60 per cent saturation, allophycocyanin was completely precipitated (P-II). The two precipitates (P-I and P-II) were separately dissolved in small amounts of water and dialyzed against water at a low temperature for 24 to 48 hours.

Further purification was performed by fractionating adsorption, using tricalcium phosphate-gel in batch-wise*. The dialyzed solutions of P-I and P-II were adsorbed with the gel at a neutral pH and the pigments adsorbed were eluted by successive extraction with a series of phosphate buffers of varied concentration and pH ($M/20$–$M/10$, pH 5.0–8.0; *cf.* the legends for Fig. 1). One of the typical results is shown in Fig. 1-A.

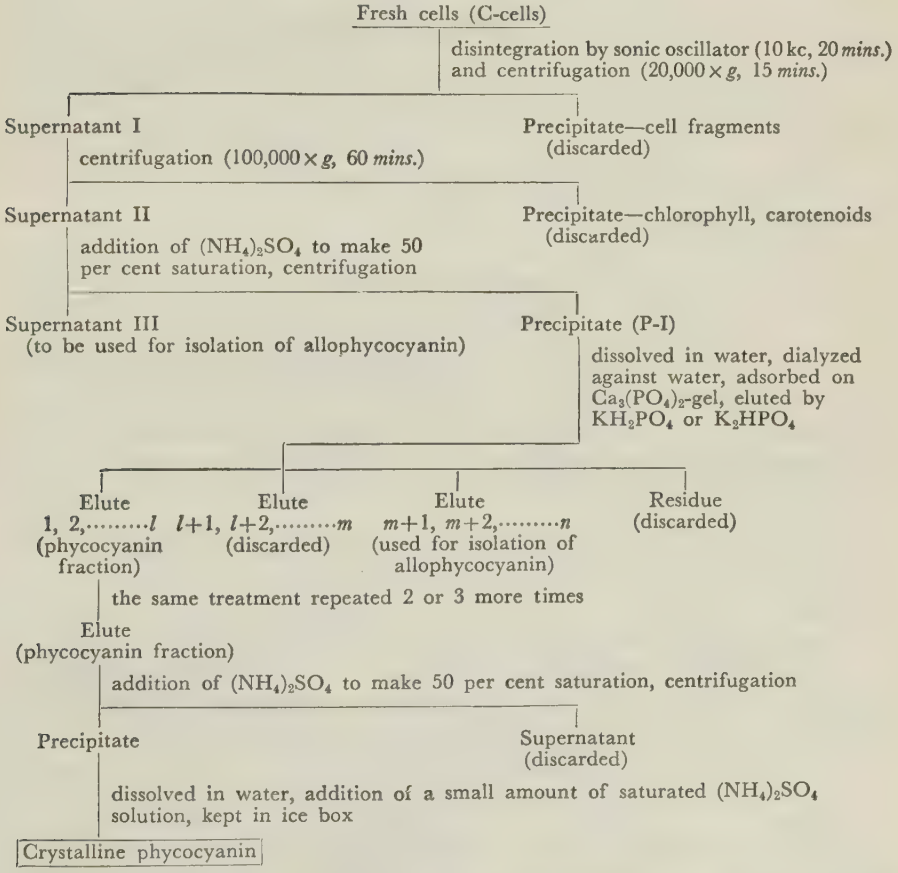
From various fractions of purified phycocyanin thus obtained, the pure phycocyanin was crystallized by ammonium sulfate precipitation. For allophycocyanin, this fractionation procedure had to be repeated 2 or 3 times in order to obtain it in a crystalline state.

Phycoerythrin from "E-cells": The procedure for preparing a crude solution of phycobilin pigments was the same as that described above. In this case, the supernatant solution

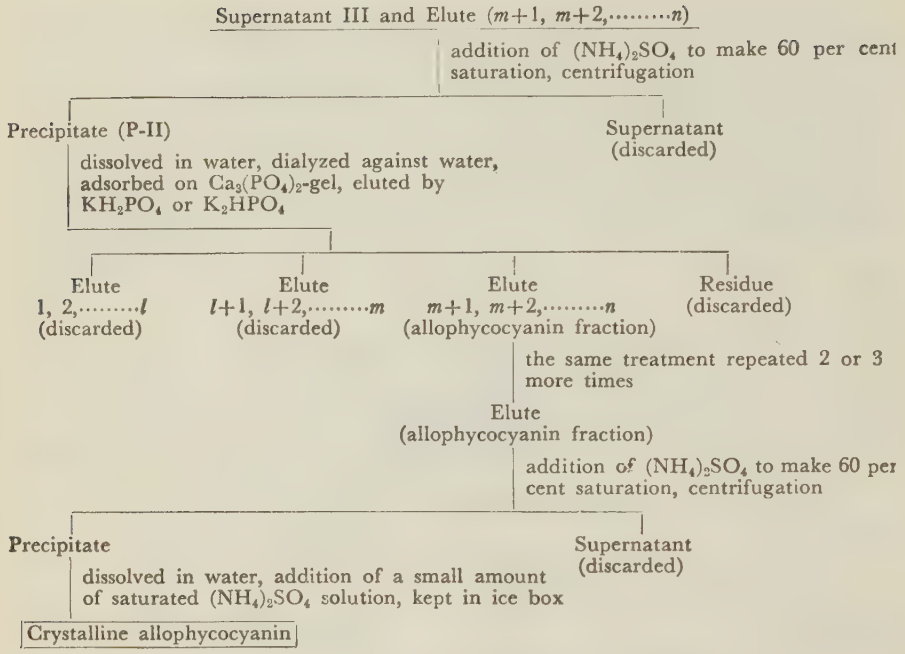
SCHEME 1

Flow-sheets of Preparation of Phycobilin Pigments

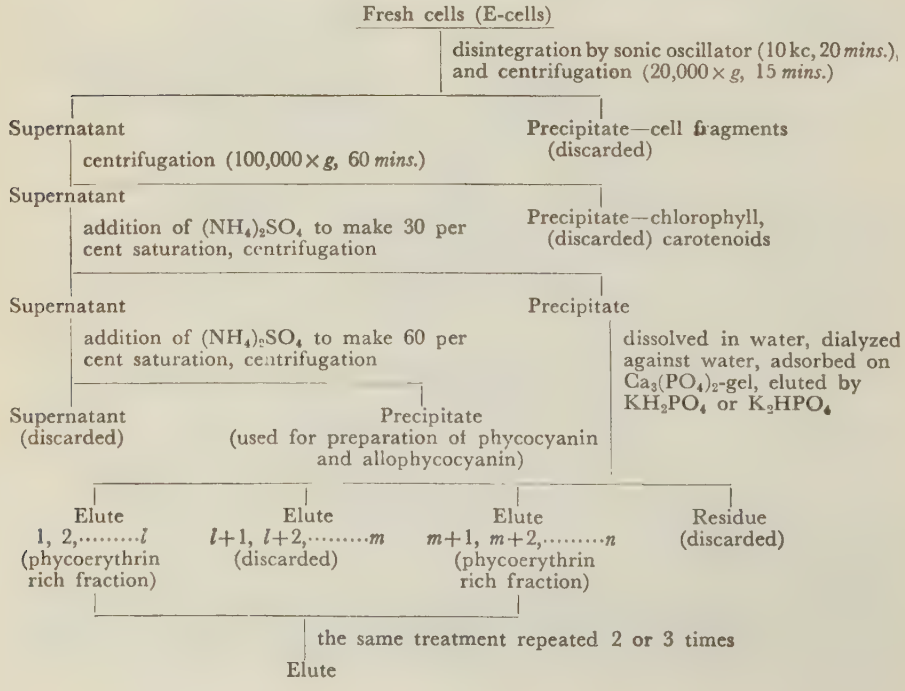
A. Preparation from C-cells

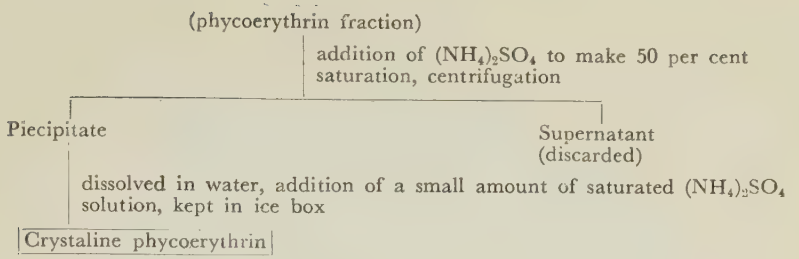


* The batch-wise use of tricalcium phosphate-gel was found to be preferable to the column-chromatographic use adopted by Haxo *et al.* (*5*), especially because of the rapidity of the adsorption procedure and the possibility of handling larger quantities of pigments at one time.



B. Preparation from E-cells





obtained by 100,000 × *g* centrifugation was reddish-purple in color. The first fractionation of phycoerythrin was achieved by 30 per cent saturation of the supernatant with ammonium sulfate, the other phycobilin pigments being left in solution. This process is indispensable for further purification of phycoerythrin, since the pigment and phycocyanin show quite similar behaviours towards tricalcium phosphate-gel treatment as will be described below. The crude phycoerythrin thus obtained was further purified by the use of calcium phosphate-gel as described above. Phycoerythrin was found to be eluted in ealier stages of extraction, closely following the disappearance of phycocyanin from the gel (Fig. 1-B). After repeating the adsorption elution procedure 2 or 3 times, a sufficiently purified solution of phycoerythrin was obtained, which was subjected to crystallization as described above.

The flow-sheet of the whole procedure is presented in Scheme 1*.

Properties of Phycobilin Pigments

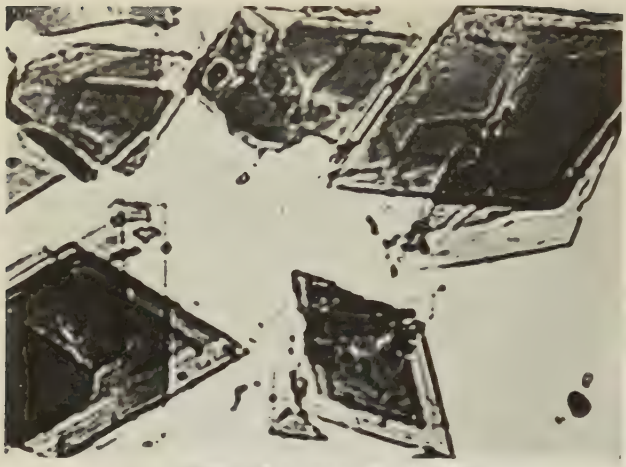
Crystalline Pigments—The crystalline phycobilin pigments finally obtained were found to be fairly homogeneous in so far as the results of electrophoretic and ultracentrifugal examinations were concerned. The spectrophotometric measurements also indicated the purity of the products.

Phycocyanin and allophycocyanin crystallized in microscopic thin platelets (Fig. 2-A and C). Phycoerythrin, on the other hand, showed the tendency to form thin needles as shown in Fig. 2-B. In some rare instances, however, platelet formation was also observed with phycoerythrin. Phycoerythrin and allophycocyanin were readily soluble in water and highly hygroscopic in nature.

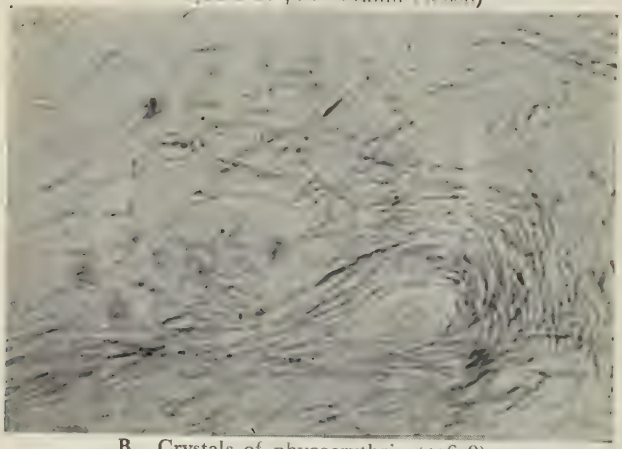
Absorption Spectrum—The absorption spectra of the phycobilin pigments were determined with a Beckman Spectrophotometer Model DK 2, using the purest samples of the pigments obtained. The results obtained are presented in Fig. 2-A, B and C. The principal absorption maxima were situated: for phycocyanin at 620 mμ, 360 mμ and 230 mμ; for phycoerythrin at 565 mμ, 380 mμ, 308 mμ and 280 mμ**, and for allophycocyanin at 650 mμ, 360 mμ and 280 mμ. It may be noted that the spectra of phycocyanin and allophycocyanin were not only very similar to each other, but they shared two smaller peaks at exactly the same position, 360 mμ and 230 mμ. These

* The same method could be applied with success to various other algae such as *Porphyra tenera* and *Polysiphonia urceolata* (*9*).

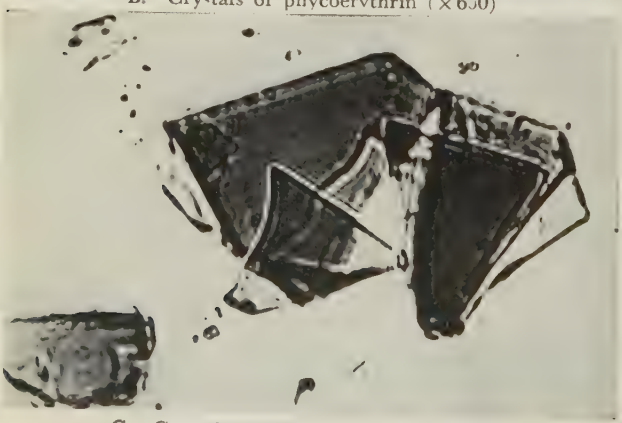
** The existence of the peak at 303 mμ in phycoerythrin was noted also in the pigment samples obtained from other sources, *e.g.*, *Porphyridium cruentum* (*8*) and *Porphyra tenera* (*9*).



A. Crystals of phycocyanin (×600)



B. Crystals of phycoerythrin (×600)



C. Crystals of allophycocyanin (×600)

FIG. 2. Microphotograms of crystalline phycobilin pigments.

two peaks for allophycocyanin has thus far not been recorded in the literature.

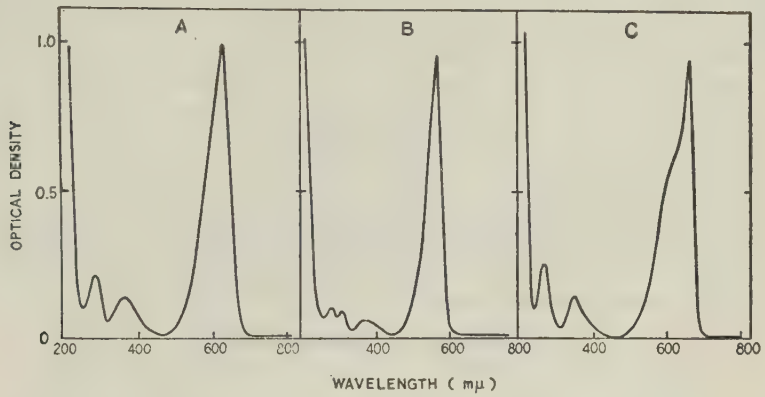


FIG. 3. Absorption spectra of phycobilin pigments. A, phycocyanin; B, phycoerythrin; C, allophycocyanin.

TABLE I

Comparison of Tolypothrix-Phycocyanin with Other Phycocyanin Preparations[1)]

Source	States	E_{620}/E_{280}	Method of extraction and purification	Reference
Tolypothrix tenuis	Cr.	4.5	Son. -Ca ph. -Am. sul.	Present work
Aphanizomenon flos aquae	Cr.	4 1	Aut. -Am. sul.	(*4*)
Synechocystis sp.	Am.	4.7	Lyo. -Am. sul.	(*10*)
Porphyra tenera	Cr	4.9	Son. -Ca ph. -Am. sul.	(*9*)
Porphyra tenera	Cr.	3.4	Aut. -Am. sul. -Riv.	(*11*)
Porphyra tenera[2)]	Cr.	2.9	Aut. -Am. sul.	(*4*)
Ceramium rubrum[2)]	Cr.	2·5	Aut. -Am. sul.	(*4*)

1) Following abbreviations are used. Column 2: Cr., crystalline; Am., amorphous, Column 4: Son., sonic disintegration; Aut., autolysis; Lyo., lyophilization; Ca ph., tricalcium phosphate-gel; Riv., rivanol treatment; Am. sul., ammonium sulfate precipitation.

2) Sample showing two absorption maxima in visible region.

The results with phycocyanin and phycoerythrin are in harmony with the findings reported by earlier investigators, who mostly worked with less purified materials. In order to compare our results with those reported by other wokers, the ratio of extinctions at 280 mμ and 620 mμ shown by various preparations of phycocyanin was calculated and listed in Table I together with the methods of preparation adopted by different workers. The low E_{620}/E_{280} values shown by some earlier preparations indicate the insufficiency of their purity even though they reported to be in crystalline forms. It is worthy of note that the phycocyanin obtained from algae of different

classes (red algae: *e.g.*, *Porphyra tenera* and blue-green algae: *e.g.*, *Tolypothrix tenuis*) show quite identical absorption spectra when they are purified to a sufficient degree. The preparations of phycocyanin which were obtained by Svedberg and Katsurai (*4*), Kitasato (*2*) and Haxo *et al.* (*5*), respectively, from *Ceramium rubrum*, *Porphyra tenera* and *Porphyra perforata*, have been reported to show two absorption peaks at 610–620 mμ and 546–555 mμ. Conceivably they were mixtures of phycocyanin (absorption maximum at 610–620 mμ) with a small amount of contaminating phycoerythrin (absorption maximum at 555–565 mμ).

Isoelectric Point—Isoelectric points were determined from the measurement of electric mobilities at various pH ranging from 3.8 to 5.7. Electric mobility was measured at 3–4° in an acetate buffer of ionic strength, $\mu=0.1$, using a Tiselius-type electrophoretic apparatus (HT-B, Hitachi, Ltd.).

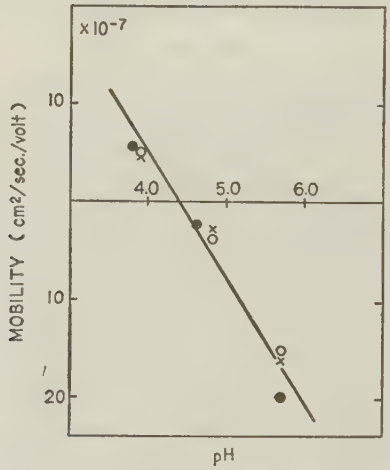


FIG. 4. Electrophoretic mobility of phycobilin pigments. Ordinate: mobility in cm².sec./volt; abscissa: pH. $\mu=0.1$; temperature: 3–4°. Open circles: phycocyanin, solid circles: phycoerythrin, crosses: allophycocyanin.

In Fig. 4 are plotted the mobilities observed in function of pH, from which it may be seen that the three pigments showed almost identical electrophoretic behaviours with isoelectric points falling equally at pH 4.4.

Molecular Size and Shape—The sedimentation constant ($s_{20,w}$)was measured with a Spinco Ultracentrifuge Model E. In Table II are presented the sedimentation constants at various hydrogen ion concentrations (phosphate buffer). In all cases, change of the pigment concentration did not lead to an appreciable shift of $s_{20,w}$ value. The $s_{20,w}$ values obtained in neutral solutions (pH 7.2–8.3) were: for phycocyanin 6.1, for phycoerythrin 10.2 and for allophycocyanin 5.9. As may be seen from the table, all pigments showed a marked decrease of $s_{20,w}$ value when pH was raised from 8.3 to 11.6. Such a change, which was found to be an irreversible process, has already been reported for phycocyanin by Svedberg *et al.* (*4*) and Eriksson-

Quensel (*12*). When the shift of pH was from 5.2 to 7.2–8.3, the $s_{20,w}$ values of phycoerythrin and allophycocyanin remained practically unchanged, while that of phycocyanin decreased to quite an appreciable extent. The change in this pH range shown by phycocyanin was found to be a reversible process.

Table II

Sedimentation Constants, $s_{20,w}$, of Phycobilin Pigments at Various pH

Measurement was carried out in *M*/20 phosphate buffer and the results were recalculated referring to the standard state. The sedimentation constants are given in Svedberg units (10^{-13} (*cm./sec.*)/(*dyne/g*) (averages of two or three determinations).

Pigment \ pH	5.2	7.2–8.3	11.6
Phycocyanin	10.9	6.1 , (2–3)*	(2.4)
Phycoerythrin	10.3	10.2 , 7.0*	(2.2)
Allophycocyanin	6.3	5.9	(1.7)

* Sedimentation constant of the additional component (see text) appearing when the pH was shifted from 5.2 to 7.2–8.3.

The figures in blackets indicate the values obtained with irreversibly modified forms of the chromoproteids.

On raising the pH from 5.2 to 8.3, there occurred conspicuous changes in the sedimentation patterns of phycocyanin and phycoerythrin; namely, there appeared additional peaks with smaller $s_{20,w}$ values which are indicated with asterisks in Table II. The appearance of these new peaks seems to indicate the splitting of the original molecule into smaller units. Detailed examination of the changes occurring in this pH range seems to be highly relevant.

The diffusion constants were computed both by the *maximum ordinate-area method*, and by the *statistical method*, using data obtained by the measurements in the Tiselius Apparatus at room temperature (13–14°) and pH 7.2 (phosphate buffer). The values of the diffusion constants obtained by the two methods coincided with each other satisfactorily, being 4.3, 4.4 and 4.3×10^{-7} cm²/sec. for phycocyanin, phycoerythrin and allophycocyanin, respectively. The results with phycoerythrin and phycocyanin were in agreement with those reported by Tiselius (*13*), who isolated the pigments from a red alga *Ceramium rubrum*.

Based on these values of sedimentation and diffusion constants and assuming the partial specific volumes of the pigment molecules to be 0.75*, their molecular weights were calculated. The results obtained are listed in Table III.

* The value actually determined by Svedberg (*4*) with phycocyanin and phycoerythrin which were isolated from *Ceramium rubrum*, *Porphyra tenera* and *Aphanizomenon flos aquae*.

TABLE III

Some Physicochemical Characteristics of Phycobilin Pigments

All figures refer to the values obtained in *M*/20 phosphate buffer, pH 7.2. For the details of determination, see text.

	Phycocyanin	Phycoerythrin	Allophycocyanin
Sedimentation constant, $s_{20,w}$ (10^{-13}(*cm./sec.*)/(*dyne/g*)	6.1	10.2	5.9
Diffusion constant, $D_{20,w}$ (10^{-7} *cm*^{2}*./sec.*)	4.3	4.4	4.3
Molecular weight, M_{sd}	138,000	226,000	134,000
Axial ratio (*a*/*b*)	8.9	1.5	9.1
Molecular extinction coefficient shown by the main peak in the spectrum (10^{11} *cm./*(*mole/liter*))	9.34	28.5	8.76

It is to be noted that the molecular weight (138,000) of *Tolypothrix*-phycocyanin was identical within the limits of experimental error with that of red algal phycocyanin (131,000) reported by Eriksson-Quensel (*12*). The molecular size of *Tolypothrix*-phycoerythrin (molecular weight: 226,000), on the other hand, was somewhat smaller than that of its red algal counterpart (molecular weight; 290,000) which was investigated by the same author. The molecular weight of allophycocyanin was estimated to be 134,000. Based on the values of $s_{20,w}$ and the diffusion constants, calculation were made of the frictional ratio (f/f_0) and further of the axial ratio (*a*/*b*) by using the figures in the table of Perrin (*14*). Also calculated were the molecular extinction coefficients by using the above-obtained values of molecular weight. The results of these calculations are summarized in Table III.

Elementary Analysis—The elementary compositions of the purified pigments are presented in Table IV. These results are somewhat different from those reported by the previous workers (*2*, *11*), especially with respect to the content of sulfur. According to Fujiwara (*11*) and Kitasato (*2*), the sulfur content of phycocyanin and phycoerythrin in always amounted to one per cent or even higher, while values as low as 0.6 (or even 0.1 in the case of phycoerythrin) have been obtained in our experiments.

Content of Phycobilin Pigments in Algal Cells—The contents in algal cells of the phycobilin pigments were computed from spectrophotometric measurements of the crude extracts obtained from fresh algal cells (Table V). As this alga contains 55 per cent protein on the basis of cell dry weight (*7*), the total content of phycobilin pigments in the cells amounts to as much as 30 per cent of the total protein. The marked difference in the phycoerythrin levels of C- and E-forms of the organism is to be noticed. It may also be

TABLE IV

Elementary Composition of Phycobilin Pigments

Element \ Pigment	Content in per cent of dry weight		
	Phycocyanin	Phycoerythrin	Allophycocyanin
C	49.14	48.57	49.67
H	7.15	7.29	7.84
N	15.33	14.82	14.46
S	0.64	0.10	0.66
Ash	0	0.37	0

TABLE V

Content of Phycobilin Pigments in Cells of Tolypothrix tenuis

	Content in per cent of dry weight of cells		
	Phycocyanin	Phycoerythrin	Allophycocyanin
C-cell[1)]	9.8	0.5	4.1
E-cell[2)]	5.9	9.0	3.5

1) Phycocyanin-rich cells grown under incandescent light.
2) Phycoerythrin-rich cells grown under fluorescent light (see text).

mentioning that allophycocyanin which has been considered to be a minor component in algal cells (*5*), was invariably discovered in amounts comparable to those of the other phycobilin pigments.

DISCUSSION

On comparing the data described above with those reported by earlier workers, it appears, likely that among the three components of phycobilin pigments at least one—and that is phycocyanin—has a definite property (regarding the molecular weight, absorption spectrum and behaviour in the sedimentation experiment) irrespective of its source, either red or blue-green algae. On the other hand, phycoerythrin from blue-green algae differs considerably from that of red algae not only in absorption spectrum and molecular weight, but also in its elementary composition. The notation of R- and C-phycoerythrin, which has been generally used to discriminate between the red pigment found in red algae and that in blue-green algae, is fully justified in this respect.

In the description of culture method of our experimental alga, we have noticed that the formation of phycoerythrin was markedly influenced by the kind of light (incandescent or fluorescent) applied during the culture. Investigations on this highly interesting phenomenon are now in progress and we shall reserve a detailed description of the result to a subsequent

paper.

SUMMARY

From the cells of a fresh-water blue-green alga, *Tolypothrix tenuis*, the three components of phycobilin pigments (phycocyanin, phycoerythrin and allophycocyanin) were isolated and purified to crystalline states by combined use of salting-out method (using ammonium sulfate) and adsorption method (using tricalcium phosphate-gel) followed by the technique of ultracentrifugation.

Using the highly purified samples, determinations were made of the various physicochemical properties of the chromoproteids, including the sedimentation and diffusion constants, electrophoretic mobilities, isoelectric points, molecular weight and elementary composition, and the results obtained were compared with those reported earlier by other workers.

The authors wish to express their gratitude to Profs. H. Tamiya, A. Takamiya and A. Watanabe, University of Tokyo, for their valuable advices during this work. Thanks are also due to Drs. T. Takeda and T. Samejima, Tokyo Institute of Technology, and Dr. K. Nagai, Institute of Infectious Diseases, University of Tokyo, who helped the writers in carrying out the measurement of sedimentation constants.

REFERENCES

(*1*) Kylin, H., *Z. phys. Chem.*, **60**, 169 (1910)
(*2*) Kitasato, Z., *Acta Phytochim.*, **2**, 274 (1925)
(*3*) Lemberg, R., *Ann. Chem.*, **461**, 46 (1928)
(*4*) Svedberg, T., and Katsurai, T., *J. Am. Chem. Soc.*, **51**, 3573 (1929)
(*5*) Haxo, F., O'hEocha, C., and Norris, P., *Arch. Biochem. Biophys.*, **54**, 162 (1955)
(*6*) Swingel, S. M., and Tiselius, A., *Biochem. J.*, **48**, 171 (1951)
(*7*) Ukai, Y., Fujita, Y., Morimura, Y., and Watanabe, A., *J. Gen. Appl. Microbiol.*, **4**, 163 (1958)
(*8*) O'hEocha, C., *Arch. Biochem. Biophys.*, 73, 207 (1958); Haxo, F., O'hEocha, C., and Strout, P., *Congr. Intern. Botan.*, Paris, Rapps. et communs, **8**, sect **17**, **35** (1954)
(*9*) Hattori, A., and Fujita, Y., *J. Biochem.*, in press
(*10*) Bannister, T. T., *Arch. Biochem. Biophys.*, **49**, 222 (1954)
(*11*) Fujiwara, T., *J. Biochem.*, **42**, 411 (1951)
(*12*) Eriksson-Quensel, I. B., *Biochem. J.*, **32**, 585 (1938)
(*13*) Tiselius, A., and Gross, D., *Kolloid Z.*, **66**, 11 (1934)
(*14*) Perrin, F., *J. phys. Radium*, (7) **7**, 1 (1936)

The Journal of Biochemistry, Vol. 46, No. 5, 1959

ACTION OF TRYPSIN ON SYNTHETIC SUBSTRATES

I. ACTION OF TRYPSIN ON MONO-, DI-, TRI- AND TETRA-GLYCYL-L-LYSINAMIDE*

By NOBUO IZUMIYA and HAKARU UCHIO

(From the Laboratory of Biochemistry, Faculty of Science, Kyushu University, Fukuoka)

(Received for publication, September 27, 1958)

The rates of hydrolysis of synthetic substrates by trypsin depend chiefly on the nature of the sensitive amino acid residue. Acylamino acid amides or esters containing L-arginine or L-lysine at the carbonyl moiety of the susceptible bond fulfil optimal structural requirement; benzoylarginine amide or methyl ester is one of the typical synthetic substrates known for the enzyme (*1*, *2*).

Although the side chain specificity of the sensitive amino acids forming the susceptible bond is a dominant factor, it should be mentioned that the hydrolytic rate of the susceptible peptide bond in a polypeptide or protein may also be influenced by the size and the spacial configuration of the peptide chain which is combined with the amino group of the sensitive amino acids. In this connection, it seemed desirable to examine the mode and rates of hydrolysis of the peptide amides $(glycyl)_n$-L-lysinamide by trypsin in which n are 1, 2, 3, and 4.

It should be noted that the previous communication from this laboratory dealing with the action of carboxypeptidase on the peptides $(glycyl)_n$-L-tyrosine in which n are 1, 2, 3, 4, and 5, has revealed that $(glycyl)_n$-L-tyrosines ($n=2\sim5$) were hydrolyzed approximately 200 times rapidly than glycyl-L-tyrosine (*3*).

EXPERIMENTAL

Enzyme

Trypsin was salt free, crystalline sample from Nutritional Biochemicals Corporation, Ohio, U.S.A. The enzyme weighed out was dissolved in 0.001 *N* HCl. The purity of the enzyme was tested by its activity towards benzoyl-L-argininamide (BzArgAm). At 25° and pH 7.8 (1/15 *M* phosphate buffer), the enzyme hydrolyzed 0.05 *M* BzArgAm with a proteolytic coefficient of 0.033. The values of 0.038 (*4*) and 0.032~0.038 (*5*) under similar experimental conditions were reported in the literatures.

Methods

The procedure was the same as that described before (*6*). The hydrolytic rates were

* The present paper was read before the 31st Annual Meeting of the Japanese Biochemical Society (Sapporo, July 1958).

followed by means of the Conway microdiffusion method (*7*). Proteolytic coefficients (C) were estimated from $C=k/e$, where $k=(1/\text{min.})\log[100/(100-\%\text{ hydrolysis})]$ and e is the protein concentration in mg. of protein N per ml. of test solution.

Synthesis of Peptide Derivatives

α-Glycyl-L-lysinamide Dihydrochloride (*GlyLysAm 2HCl*)—α-Carbobenzoxyglycyl-ε-carbobenzoxy-L-lysinamide (0.005 mole) (*8*) suspended in 0.2 *N* methanolic HCl (52.5 ml.) was treated with dry hydrogen in the presence of palladium black. The suspended crystals were dissolved gradually during the hydrogenation. The filtrate from the catalyst was evaporated *in vacuo*. Trituration of the residue with ether resulted in crystallization of the desired compound. Yield, 86 per cent; m. p. 203–206° (decomp.); $[\alpha]_D^{22}-12.0°$ (c 1, in water).

$C_8H_{20}O_2N_4Cl_2$ (275.2) Calcd. C 34.9, H 7.3, N 20.3
Found C 35.2, H 7.4, N 20.0

Glycyllysinamide dihydrobromide has been obtained previously (*9*).

α-Carbobenzoxydiglycyl-ε-carbobenzoxy-L-lysine Methyl Ester—The mixed anhydride of carbobenzoxydiglycine (0.01 mole) in tetrahydrofuran (20 ml.) was coupled with a mixture of ε-carbobenzoxy-L-lysine methyl ester hydrochloride (0.01 mole) (*8*), triethylamine (0.01 mole), and chloroform (20 ml.) in the usual manner (*3*, *6*). The reaction mixture was left overnight, and the crystals appeared after washing the mixture with water, 4 per cent bicarbonate solution, 2 per cent HCl, and water, successively, by means of decantation. It was collected and recrystallized from ethyl acetate-ether-petroleum ether. Yield, 85 per cent; m. p. 84°; $[\alpha]_D^{25}-2.2°$ (c 2, dimethylformamide (DMF)).

$C_{27}H_{34}O_8N_4$ (542.6) Calcd. N 10.3
Found N 10.1

α-Carbobenzoxydiglycyl-ε-carbobenzoxy-L-lysinamide—The above ester (0.005 mole) was dissolved in 50 ml. of methanol previously saturated with dry NH_3 at 0°, and the solution was kept at room temperature for 2 days. The solution was then concentrated *in vacuo*. The crystalline residue was recrystallized from hot methanol. Yield, 82 per cent; m. p. 179°; $[\alpha]_D^{22}-3.0°$ (c 2, in DMF).

$C_{26}H_{33}O_7N_5$ (527.6) Calcd. N 13.3
Found N 13.3

α-Diglycyl-L-lysinamide Dihydrochloride (*Gly$_2$LysAm 2HCl*)—The compound was prepared in non-crystalline powder from the above amide as described in the case of GlyLysAm. Yield, 87 per cent; $[\alpha]_D^{22}-23.6°$ (c 1, in water).

$C_{10}H_{23}O_3N_5Cl_2\cdot 1/2H_2O$ (341.3) Calcd. C 35.1, H 7.1, N 20.5
Found C 35.1, H 7.2, N 20.2

α-Carbobenzoxytriglycyl-ε-carbobenzoxy-L-lysine Methyl Ester—The mixed anhydride of carbobenzoxytriglycine in dimethylformamide (*3*) was coupled with carbobenzoxylysine methyl ester as described in the case of the corresponding diglycyl derivative. The crude product obtained was recrystallized from acetone-petroleum ether. Yield, 57 per cent; m. p. 129°; $[\alpha]_D^{25}-3.0°$ (c 2, in DMF)

$C_{29}H_{37}O_9N_5$ (599.6) Calcd. N 11.7
Found N 11.5

This compound has been obtained previously by the coupling of carbobenzoxydiglycine and α-glycyl-ε-carbobenzoxylysine methyl ester (*10*).

α-Carbobenzoxytr.glycyl-ε-carbobenzoxy-L-lysinamide—The compound was prepared from the above ester in the same manner as that for the corresponding diglycyl derivative. Yield,

81 per cent; m.p. 165–166°; $[\alpha]_D^{22}$ −3.2° (c 2, in DMF).

$C_{28}H_{36}O_8N_6$ (584.6) Calcd. N 14.4
Found N 14.6

α-Triglycyl-L-lysinamide Dihydrochloride (*Gly_3LysAm 2HCl*)—The compound was prepared in hygroscopic crystalline form from the above amide in the usual manner. Yield, 73 per cent; $[\alpha]_D^{22}$ −20.1° (c 1, in water).

$C_{12}H_{26}O_4N_6Cl_2 \cdot 1/2H_2O$ (398.3) Calcd. C 36.2, H 6.8, N 21.1
Found C 36.4, H 7.2, N 20.7

α-Carbobenzoxytetraglycyl-ε-carbobenzoxy-L-lysine Methyl Ester—The mixed anhydride of carbobenzoxytetraglycine (*3*) was applied as described in the case of the corresponding diglycyl derivative. The crude product was recrystallized from methanol-ether. Yield, 81 per cent; m.p. 171°; $[\alpha]_D^{25}$ −3.6° (c 2, in DMF).

$C_{31}H_{40}O_{10}N_6$ (656.7) Calcd. N 12.8
Found N 12.7

α-Carbobenzoxytetraglycyl-ε-carbobenzoxy-L-lysinamide—The above ester was amidated using a large amount of methanolic NH_3 at 40°. The crude product was recrystallized from a large amount of hot methanol. Yield, 67 per cent; m.p. 177–179°; $[\alpha]_D^{22}$ −2.8° (c 2, in DMF).

$C_{30}H_{39}O_9N_7$ (641.7) Calcd. N 15.3
Found N 15.5

α-Tetraglycyl-L-lysinamide Dihydrochloride (*Gly_4LysAm 2HCl*)—The compound was prepared in crystalline form from the above amide. The product was recrystallized from methanol. It was dried over P_2O_5 *in vacuo* at 70°. Yield, 65 per cent; $[\alpha]_D^{22}$ −24.1° (c 1, in water).

$C_{14}H_{29}O_5N_7Cl_2 \cdot H_2O$ (464.4) Calcd. C 36.4, H 6.5, N 21.1
Found C 36.7, H 6.8, N 20.1

RESULTS AND DISCUSSION

pH-Activity Curve of GlyLysAm—Since no experiment concerning the optimum pH with an aminoacyl arginine or lysine derivative for trypsin had been reported, comparative measurements of the effect of pH on the amidase activity of trypsin were made with GlyLysAm as a representative substrate, and the results were shown in Fig. 1, an optimum pH of the reaction appearing to be near 8.2. For comparison, data for BzArgAm were included in Fig. 1, and an optimum pH of the reaction appeared to be near 7.8. The optimum pH values of 7.8 (*11*) and 8.0 (*12*) were reported in the literatures.

It is interest that the optimum pH of GlyLysAm is slightly higher than that of BzArgAm. This results may be explained by the fact that the rates of the reaction will depend on the concentration of available substrate at a given pH as described already (*3*, *6*, *13*).

Proteolytic Coefficients in Various Initial Substrate Concentrations—In order to compare the hydrolytic rates of the peptide amides Gly_nLysAm (n=1∼4) by trypsin, the values of proteolytic coefficients in various substrate concentrations and C_{max} were determined. For comparison, the values for BzArgAm were also determined.

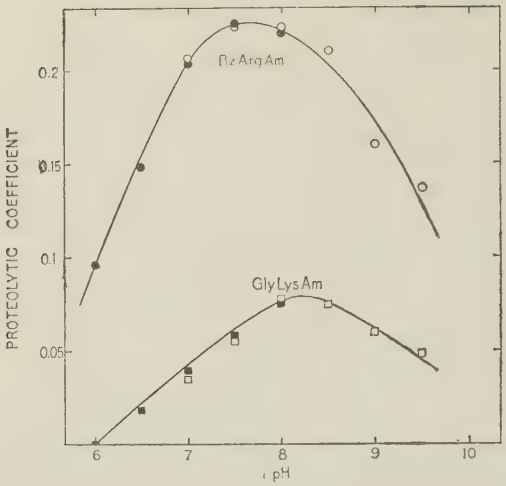


FIG. 1. The pH dependence of the hydrolysis of BzArgAm and GlyLysAm by trypsin at 30°. The substrate concentration was 0.01 *M*. ●, ■; 0.1 *M* phosphate buffer. ○, □; 0.1 *M* with respect to the amine component of Tris-HCl buffer.

The compounds were tested at 0.025 *M*, 0.015 *M*, 0.01 *M* and 0.005 *M* substrate concentrations. Representative data for the hydrolysis of GlyLysAm at 0.025 *M* and 0.01 *M* substrate concentrations are shown in Table I as an example. Under the conditions given in the section of *methods*, it was found

TABLE I

Example in Measurements of Hydrolysis of GlyLysAm by Trypsin

pH 8.0 (0.1 *M* phosphate buffer); enzyme concentration, 0.045 mg. protein N/ml.; temperature, 30°.

Substrate concentration (*M*)	Time (*min.*)	Hydrolysis (*per cent*)	Proteolytic coefficient
0.025	11	5.7	0.051
	20	9.5	0.048
	31	14.3	0.048
	39	18.0	0.049
	47	20.7	0.048
Average			0.049
0.01	10	7.7	0.077
	19	14.2	0.078
	29	20.1	0.075
	37	25.5	0.077
	46	30.5	0.076
Average			0.077

that the hydrolysis of the substrates tested followed first order kinetics within the extent of error in all cases. A summary of all coefficients determined are given in Table II.

TABLE II

Proteolytic Coefficients of BzArgAm and Gly_nLysAm in Various Initial Substrate Concentrations

pH 8.0 (0.1 *M* phosphate buffer) temperature, 30°.

Substrate	Proteolytic coefficient			
	0.025 *M*	0.015 *M*	0.01 *M*	0.005 *M*
BzArgAm	0.096	0.147	0.22	0.33
GlyLysAm	0.049	0.063	0.077	0.10
Gly_2LysAm	0.81	1.20	1.56	2.0
Gly_3LysAm	0.80	1.14	1.47	1.99
Gly_4LysAm	0.174	0.27	0.37	0.64

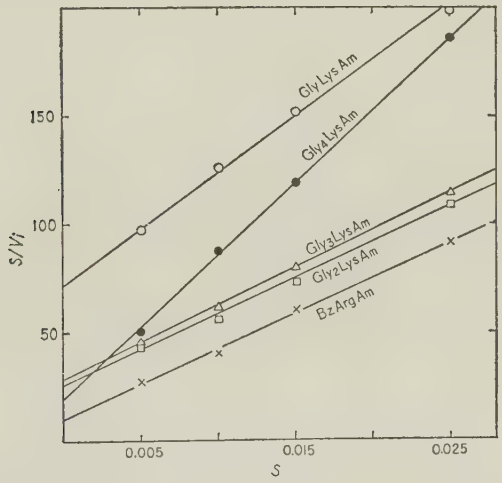


FIG. 2. The plots of initial substrate concentration S divided by initial velocity V_i *versus* S for the hydrolysis of the substrates at 30° and pH 8.0. The enzyme concentrations were 0.0496 mg. (BzArgAm), 0.045 mg. (GlyLysAm), 0.00495 mg. (Gly_2LysAm), 0.00491 mg. (Gly_3LysAm), and 0.01347 mg. protein N per ml. (Gly_4LysAm).

Reaction Kinetics and C_{max}—Measurements of initial rates of hydrolysis at different concentrations of BzArgAm and Gly_nLysAm ($n=1\sim4$), and the use of the plotting method of Lineweaver and Burk, gave the values of $K_m=(k_2+k_3)/k_1$ at pH 8.0 and 30° for the hydrolysis of the substrates by trypsin (*3*, *6*). The magnitudes of k_3 defined as the rate constants for the conversion of enzyme-substrate complex to enzyme and products were also

determined by plots of Lineweaver and Burk. Plots for the hydrolysis of the substrates are shown in Fig. 2. The maximum proteolytic coefficients (C_{max}) are calculated by the equation of $C_{max} = k_3/2.3\,K_m$ (*1*). The values of K_m, k_3 and C_{max} measured are shown in Table III.

Table III

Kinetic Constants of BzArgAm and Gly_nLysAm

pH 8.0 (0.1 *M* phosphate buffer); temperature, 30°.

Substrate	K_m (10^{-3} *M*)	k_3* (10^{-3})	C_{max}
BzArgAm	3.0	6.2	0.90
GlyLysAm	1.43	4.4	0.134
Gly_2LysAm	7.9	62	3.4
Gly_3LysAm	8.5	62	3.2
Gly_4LysAm	2.8	11.2	1.74

* In *M*/liter/min./mg. protein N/ml.

As seen from Table II and III, the sizes of the peptide chains which are attached to α-amino group of lysinamide have the remarkable influence on the hydrolysis of the substrates Gly_nLysAm (n=1~4); Gly_2LysAm is hydrolyzed at the highest rate among the four kinds of the substrates. This results may suggest that hydrolytic rate of a susceptible peptide bond in a polypeptide or protein by trypsin is influenced to a certain extent by the length of peptide chain which is combined with the amino group of the sensitive amino acids, arginine or lysine.

It would be of interest to note that no difference in the hydrolytic rates by carboxypeptidase of the substrates $(glycyl)_n$-L-tyrosine in which n are 2, 3, 4 and 5 were observed in this laboratory (*3*).

Hofmann and Bergmann reported that benzoylglycylargininamide is hydrolyzed 10.5 times rapidly than benzoylargininamide at 0.05 *M* substrate concentration (*4*). In the present investigation, it was also observed that the introduction of the additional glycyl residue to GlyLysAm causes a marked increase in the hydrolytic rate; Gly_2LysAm was hydrolyzed 17 times rapidly than GlyLysAm at 0.025 *M* substrate concentration.

Paper Chromatography of Reaction Mixture—In addition to measurements by Conway method of the amount of NH_3 liberated, the reaction was also followed by transferring 5~20 μl. samples at various times on filter paper. In all cases, the substrates are hydrolyzed rapidly by trypsin to the corresponding Gly_nLys (n=1~4) and NH_3, occurring no transpeptidation reaction. The R_f values of the reference compounds in two different solvent systems are given in Table IV.

Levin *et al.* have reported already that GlyLysAm is solely hydrolyzed by trypsin to GlyLys and NH_3 (*9*).

Table IV

*R_f Values of Reference Compounds**

The compounds were chromatographed on Toyo Roshi No. 50 paper using *n*-butanol : acetic acid : pyridine : water (15 : 3 : 10 : 12, by volume) and *n*-butanol : acetic acid : water (4 : 1 : 2, by volume). The ascending technique was applied.

Substance	R_f	
	(15 : 3 : 10 : 12, by vol.)	(4 : 1 : 2, by vol.)
GlyLys	0.11	0.06
GlyLysAm	0.14	0.10
Gly_2Lys	0.10	0.04
Gly_2LysAm	0.13	0.08
Gly_3Lys	0.10	0.04
Gly_3LysAm	0.11	0.06
Gly_4LysAm	0.10	0.04

* The synthesis of $(glycyl)_n$-L-lysine in which n are 1, 2 and 3 has been described in the communication from this laboratory (*10*).

SUMMARY

1. A number of $(glycyl)_n$-L-lysinamide dihydrochlorides in which n are 1, 2, 3 and 4 have been synthesized and tested as substrates for trypsin.

2. By the use of glycyl-L-lysinamide, the pH optimum of hydrolysis was found to be near 8.2. For comparison, the pH optimum for benzoyl-L-argininamide was checked and found to be near 7.8.

3. The values of proteolytic coefficients at the initial substrate concentrations of 0.025 *M*, 0.015 *M*, 0.01 *M*, and 0.005 *M*, and C_{max}, determined at 30° and pH 8.0, were taken as measures of the relative susceptibility of hydrolysis of the substrates by trypsin. It was determined that the order of susceptibility to hydrolysis is listed as: diglycyl-≧triglycyl->tetraglycyl-> monoglycyl-L-lysinamide.

4. Chromatographic analysis of the reaction mixture proved the simple hydrolysis of the substrates to the corresponding $(glycyl)_n$-L-lysine and NH_3.

The authors wish to thank Prof. S. Shibuya for his interest, and Dr. A. Tanaka for his discussion in this study. The authors also thank Dr. J. P. Greenstein, Dr. M. Winitz and Dr. S. M. Birnbaum for generous gift of the amino acids and the enzyme.

REFERENCES

(*1*) Neurath, H., and Schwert, G. W., *Chem. Rev.*, **46**, 69 (1950)
(*2*) McDonald, C. E., and Balls, A. K., *J. Biol. Chem.*, **229**, 69 (1957)
(*3*) Izumiya, N., and Uchio, H., *J. Biochem.*, **46**, 235 (1959)
(*4*) Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **138**, 243 (1941)

(5) Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E., *J. Biol. Chem.*, **172**, 221 (1948)
(6) Izumiya, N., and Yamashita, T., *J. Biochem.*, **46**, 19 (1959)
(7) Johnston, R. B., Mycek, M. J., and Fruton, J. S., *J. Biol. Chem.*, **185**, 629 (1950)
(8) Bergmann, M., Zervas, L., and Ross, W. F., *J. Biol. Chem.*, **111**, 245 (1935)
(9) Levin, Y., Berger, A., and Katchalski, E., *Biochem. J.*, **63**, 308 (1956)
(10) Uchio, H., and Izumiya, N., *Memoirs of the Faculty of Science, Kyushu Univ., Ser. C*, **3**, 5 (1958)
(11) Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127**, 643 (1939)
(12) Shukuya, R., and Watanabe, K., *J. Biochem.*, **44**, 481 (1957)
(13) Izumiya, N., and Yamashita, T., *J. Biochem.*, **45**, 337 (1959)

The Journal of Biochemistry, Vol. 46, No. 4, 1959

A PROTEOLYTIC ENZYME OF STREPTOMYCES GRISEUS

I. PURIFICATION OF A PROTEASE OF STREPTOMYCES GRISEUS*

By MASAO NOMOTO and YOSHIKO NARAHASHI

(From the Institute of Physical and Chemical Research, Tokyo)

(Received for publication, October 1, 1958)

Purification of protease of bacteria or fungi has been reported by many investigators, but so far there is little information about the protease of *Actinomycetes*, a large family of microorganisms widely distributed in nature.

Recently, it was found by the authors that a strain of *Streptomyces griseus* which has been used for the industrial production of streptomycin excreted a remarkable amount of protease in the usual culture broth, together with streptomycin. The protease productivity of this strain was almost equal to that of excellent strains of *Bacillus subtilis* which have been used for industrial production of bacterial protease. Up to date, however, the existence of this enzyme has not been known, and the enzyme has been completely destroyed under the severe condition during conventional procedures for the purification of streptomycin.

Thus, it was attempted to utilize this enzyme as a by-product of streptomycin production in order to reduce the cost of manufacturing this antibiotic. After many trials, this protease could be separated from streptomycin by a salting out procedure and was highly purified by the following procedures; resin column treatment, salting out and acetone precipitation. Crystalline preparations of the protease are obtainable through these procedures. This enzyme was named as "Streptomyces protease G" by the authors.

In this paper is presented the method for separation and purification of this protease.

EXPERIMENTALS

(*I*) *Assay*

Assay for Protease Activity—Hagihara's method (*1*) based on the methods by Anson (*2*) and Kunitz (*3*) was employed with a little modification as follows: 1 ml. of enzyme solution suitably diluted with *M*/15 sodium phosphate buffer (pH 7.4) was mixed with 1 ml. of 2.0 per cent casein solution (pH 7.4) and allowed to react for 10 minutes at 40°. The reaction mixture was treated with 2 ml. of protein precipitation reagent (containing 0.1 *M* CCl_3COOH, 0.2 *M* CH_3COOH and 0.2 *M* CH_3COONa) and kept for 20 minutes

* Most of these data were presented at General Meeting of the Japanese Biochemical Society at Tokyo University in November 1955.

at 40°. The resulting precipitates were removed by filtration. To 1 ml. of this filtrate was added 5 ml. of 0.4 *M* sodium carbonate and 1 ml. of diluted (1/5) Folin-Ciocalteu's reagent (*4*) and the mixture was kept for 20 minutes at 40°. The optical density (OD) of the resulting colored solution was measured at 670 mμ. and the readings were corrected for the value of blanks, in which enzyme solution was mixed with the protein precipitation reagent before the casein solution was added.

As shown in Fig. 1, a good parallel relation between the enzyme activity (concentration) and the OD value was obtained in a certain range of enzyme concentration.

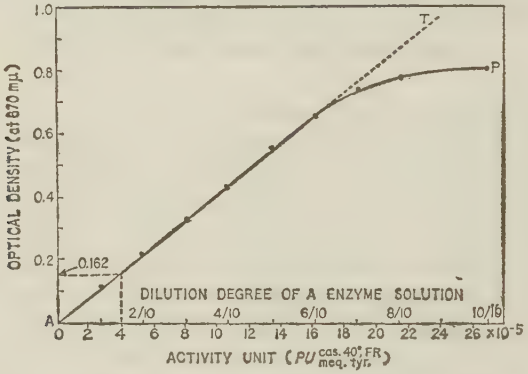


FIG. 1. Standard curve for assaying *Streptomyces griseus* protease by the casein-Folin color method.

Activity unit was calculated from measured OD value as follows, after correcting by tangent line AT;

$$PU^{\text{cas. 40°, FR}}_{\text{m. eq. tyr.}}/\text{ml.} = \frac{\text{OD } (measured)}{\text{OD } (1\ m.\ eq.\ tyrosine)} \times \frac{\text{volume } (ml.)}{\text{reaction time } (min.)}$$

$$= \frac{\text{OD } (measured)}{1620} \times \frac{4}{10} = \text{OD } (measured) \times 2.469 \times 10^{-4}$$

One unit of protease activity was defined as the activity of enzyme when it liberated the digestion product which was not precipitated with the above reagent and gave the Folin color equivalent to 1 milli-equivalent of tyrosine, per 1 minute's reaction at 40°. The unit was determined by the standard curve in Fig. 1, and represented by $PU^{\text{cas. 40°, FR}}_{\text{m. eq. tyr.}}$ (*Abbreviation*: *PU*).

Assay for Nitrogen Concentration—Nitrogen concentration was determined by micro-Kjeldahl method, or conveniently, by measuring the OD value of Folin color of samples at 670 mμ. In the latter case, the nitrogen concentration was represented as *PN*, after converting the OD value to the quantity of m. eq. of tyrosine which gave the same Folin color, as follows;

$$PN/\text{ml.} = \frac{\text{OD } (measured)}{\text{OD } (1\ m.\ eq.\ tyrosine)} = \frac{\text{OD } (measured)}{1620} = \text{OD } (measured) \times 6.173 \times 10^{-4}$$

(*II*) *Materials*

Materials of Enzyme—Usual culture broth of *Streptomyces griseus* supplied from the Kaken Chemical Co. Ltd., was used. As shown in Fig. 2, the activity of protease excreted in

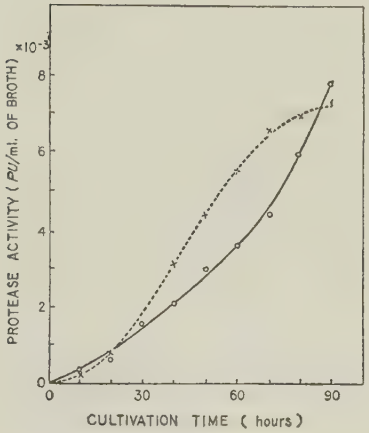


FIG. 2. Typical production curve of protease in usual culture broth of *Streptomyces griseus*. —○— protease production, —×— streptomycin production.

TABLE I

Comparison of Protease Productivity of Some Microorganisms Commercially Used

Strain	*PU*/ml. of culture broth
Streptomyces griseus[1]	$7\text{–}10\times10^{-3}$
Streptomyces aureofaciens[2]	$0.15\times$ „
Streptomyces venezuelae[3]	$0.18\times$ „
Streptomyces hachijoencis[4]	$0.8\times$ „
Bacillus subtilis-N[5]	$7\text{–}10\times$ „
Bacillus subtilis-N'[5]	$7\text{–}10\times$ „
Bacillus subtilis-R[5]	$6\text{–}8\times$ „

1) Commercial culture broth supplied from Kaken Chemical Co., Ltd.

2) Submerged culture, for 72 hours at 25°. Medium: 25 g. of starch, 35 g. of soybeen meal, 5 g. of dried yeast, 2 g. of corn steep liqure, 2.5 g. of NaCl, 3 g. of $CaCO_3$ per 1 liter of water.

3) Submerged culture, for 72 hours at 25°. Medium: 20 g. stareh, 10 g. of meat extracts, 5 g. of peptone, 10 g. of NaCl per 1 liter of water.

4) Submerged culture, for 72 hours at 25°. Medium: 25 g. of starch, 35 g. of soybeen meal, 5 g. of dried yeast, 3.5 g. of $CaCO_3$ and trace of salts per 1 liter of water.

5) Commercial culture broth supplied from Nagase & Co., Ltd. or submarged culture, for 48 hours at 30° in glucose-soybeen meal medium. Stock cultures of these strains were obtained from Prof. K. Okunuki.

the culture broth reaches approximately maxima after the cultivation period of 90-96 hours.

Protease productivities of some microorganisms employed in commercial use were compared with that of *Streptomyces griseus* (Table I). From these results, it was proved that *Streptomyces griseus* possessed a remarkable protease productivity comparable to that of *Bacillus subtilis* strains *N*, *N'*, *R* which are used for the industrial production of bacterial amylase and protease. Other strains of *Actinomycetes* such as *Streptomyces aureofacience*, *Streptomyces venezuelae* and *Streptomyces hychijoensis*, which are employed for the industrial production of aureomycin (chlortetracycline), chloramphenicol and sarcomycin respectively, produced no appreciable amount of protease. Therefore, this strain of *Streptomyces griseus* seems to be useful for the commercial production of protease.

Moreover, amylase productivity of this strain assayed by modified Wohlgemuth's method (*5*) was almost negligible, so that the purification method of this protease was fortunately not troubled with contamination of amylase.

Selection of Resins—Recently, a few kinds of ion exchange resin have been successfully employed for the purification of enzymes. Especially, Hagihara *et al.* (*6*) pointed out that Duolite C-10, a high porous sulfonic acid type cation exchange resin, was the most valuable tool for the purification of a bacterial protease. Therefore, it would be expected that these techniques were also useful for the purification of our protease. Then, in order to choose the resin suitable for the present purpose, a screening test was performed.

TABLE II

Comparison of Adsorption Capacity of Various Kinds of Resin

Each 2 g. of resin (60-80 mesh) was buffered at pH 6.0 and put in a column, 12 mm. in diameter. Enzyme solution was passed through this column. Apparent adsorption capacity of resin for the enzyme was calculated as follows: *PU* adsorbed = *PU* of original solution − *PU* of effluent.

Resin	Apparent adsorption capacity of resin (*PU*/*g*.)
Kaken C-1 (SP-3)	3.27×10^{-1}
Duolite C-10	2.56× „
Amberlite IRC-50	3.02× „
Duolite CS-101	3.05× „
Kaken Phenol-type	3.19× „
Duolite S-30	2.84× „
Duolite A-2*	0.05× „
Duolite A-7*	0.10× „
Amberlite IRA-410*	0.22× „
Amberlite IR-4B*	0.12× „

* These anion exchangers were used in OH-form.

In such a case, it is generally recommended to condition the resin with the more acidic and diluted buffer solution and to elute the adsorbed substances with the more basic and concentrated buffer solution. For instance, Hagihara *et al.* (*1*, *6*) reported that the adsorption capacity of cation exchange resin for the bacterial protease was much affected by the pH of the resin column and became larger at a lower pH. Therefore, in this experiment, the adsorption capacity of resins was compared at pH 6.0, as follows:

Each 2 g. of resin (60–80 mesh at swelled H-form) was buffered at pH 6.0 with *M*/15 sodium phosphate buffer (pH 6.0) and put into a column, 12 mm. in diameter. Through this resin column, about 50 ml. of enzyme solution (pH 6.0) was passed at the rate 0.2 ml. per minute. Adsorption capacity of resin was calculated as the difference in the total activity

TABLE III

Comparison of Elution Yield with Various Eluting Solutions

The protease adsorbed on resin column was eluted by passing the eluting solution.

Resin	Eluting solution	Average yield (%)
Kaken C-1 (SP-3)	*M*/2 Sodium borate buffer* (pH 9.0–9.4)	50–70
	M/2 Sodium borate buffer (pH 9.2)+5% NaCl	50–70
	M/2 Sodium carbonate buffer (pH 9.0)	30–40
	M/2 Sodium acetate (pH 9.0)+3% NaCl	40–60
	30% Acetone Solution	3
	Distilled water	0– 1
Duolite C-10	*M*/2 Sodium borate buffer (pH 9.0–9.4)	50–70
	M/2 Sodium borate buffer (pH 9.2)+5% NaCl	50–70
	M/2 Sodium acetate (pH 9.0)+3% NaCl	40–60
	30% Acetone solution	2
	Distilled water	0– 2
Kaken Phenoltype	*M*/2 Sodium borate buffer (pH 9.0)	0
	50% Acetone solution (pH 5.6)	40–60
	50% Acetone solution (pH 8.0)	40–60
	30% Acetone solution (pH 5.6)	30–50
	50% Ethanol solution (pH 5.6)	40–60
	50% Methanol solution (pH 5.6)	20–30
	Distilled water	1– 3
Duolite S-30	*M*/2 Sodium borate buffer (pH 9.0)	30–35
	M/2 Sodium acetate (pH 9.0)+3% NaCl	5–15
	M/2 Sodium phosphate (pH 9.0)	5–15
	M/5 Sodium borate buffer (pH 9.0)+30% acetone	50–70
	M/5 Sodium acetate (pH 9.0)+30% acetone	50–70
	M/5 Sodium phosphate (pH 9.0)+30% acetone	50–70
	30% Acetone solution (pH 7.4)	20–30
	40% Acetone solution (pH 7.4)	25–35
	Distilled water	5
Amberlite IRC-50 and Duolite CS-101	*M*/2 Sodium borate buffer (pH 90)	0–10
	M/2 Sodium acetate (pH 9.0)	0–10
	M/2 Ammonium phosphate buffer (pH 9.0)	0–10
	10% Acetone solution (pH 7.4)	0–10

* Clark and Lubs' sodium borate buffer containing 0.5 *M* KCl.

between the original enzyme solution and the effluent. The results are shown in Table II.

The enzyme adsorbed on resin was then eluted by passing about 30 ml. of eluting solution containing a suitable amount of salts or organic solvent at the rate of 0.1 ml. per minute (Table III).

From the results of screening test, it appears that Kaken C-1 (SP-3) resin* (*7*) is the most excellent in respect to adsorption capacity and elution efficiency. Duolite C-10, Duolite S-30** or Kaken Phenol-type resin*** was also proved to be useful for the present purpose.

However, Kaken Phenol-type resin was found to have an exceptional property, *i.e.*, its adsorption capacity for our protease was not affected by the pH of resin column (Table IV). Moreover, the adsorbed enzyme on this resin could be eluted merely with

Table IV

Comparison of Adsorption Capacity of Some Resins for the Protease at Various pH

Each 2 g. of resin (60–80 mesh at swelled H-form) was buffered at various pH with diluted buffer and put into a column, 12 mm. in diameter. Enzyme solution was passed through this column. Apparent adsorption capacity of resin for the enzyme was calculated as described in Table II.

Resin	Apparent adsorption capacity of resin (*PU/g.*)		
	H-form	pH 6.0	pH 9.0
Kaken Phenol-type	3.18×10^{-1}	3.19×10^{-1}	3.17×10^{-1}
Duolite S-30	$2.85\times$ „	$2.84\times$ „	$2.84\times$ „

organic solvents such as acetone, ethanol or methanol, but not with the usual alkaline buffer solution (Table III). Addition of salts or alteration of pH for organic solvents had no influence upon the elution efficiency. Besides these characteristics, this resin has another valuable property in that it adsorbs no coloring matter. Thus, the treatment with this resin easily resulted in the colorless enzyme solution. However, since the adsorption capacity of this resin was readily reduced by the regeneration procedure, it is desirable to improve this defect for practical employment.

The property of Duolite S-30 to adsorb and release our protease seemed to be intermediate of that of Kaken Phenol-type resin and that of cation exchange resins, as shown in Tables III and IV.

Either Duolite CS-101 or Amberlite IRC-50, both having merely carboxyl group as a functional group, showed apparently a remarkable adsorption capacity for the protease, but the enzyme adsorbed on resins could hardly be eluted by any means.

Some kinds of anion exchange resin, such as Duolite A-2, Duolite A-7, Amberlite IRA-410 or Amberlite IR-4B, were useless for our purpose. However, the former two were proved to be useful for the elimination of coloring matter, as mentioned by Hagihara

* A high porous, salicylic acid type cation exchange resin, specially prepared by H. Kawabe *et al.*, the Institute of Physical and Chemical Research, Tokyo, Japan.

** A phenolic matrix, resinous absorbent with mixed polar functional group.

*** A high porous, phenolic resinous adsorbent having no functional group as ion exchanger. This resin was also prepared by H. Kawabe *et al.*

et al. (*8*).

(*III*) *Methods*

Preparation of the Enzyme Solution from the Culture Broth—The mycelia of *Streptomyces griseus* existing abundantly in the culture broth were first removed by centrifugation at $3,000 \times g$ About 9 litres of dark-brownish supernatant was obtained from 12 litres of the original broth.

To 9,000 ml. of the above supernatant, 540 ml. of 0.5 *M* Na_2HPO_4 solution and 180 ml. of 2 *M* $CaCl_2$ solution were added successively at pH 7.8. After setting the mixture for 4 hours below 10°, the resulting precipitates of calcium phosphate gel were centrifuged off. Thus, about 10,500 ml. of yellowish supernatant (including the washings with water) was obtained with about 86 per cent recovery of the protease activity.

Salting Out Procedure (*Separation of Protease and Streptomycin*)—To 10,500 ml. of the enzyme solution, prepared with the above calcium phosphate gel method, about 6,000 g. of ammonium sulfate was added with stirring at pH 6.0. After setting the mixture overnight at 4°, the resulting precipitate was collected by centrifugation at $3,000 \times g$ for 20 minutes. The cake of enzyme precipitate was well suspended in water and insoluble materials were again centrifuged off. Thus, 3,400 ml. of clear yellowish enzyme solution was obtained and about 90 per cent of the protease activity was detected in it with three-fold purification. On the other hand, streptomycin was entirely discovered in the supernatant separated from enzyme precipitate.

Then, the clear enzyme solution obtained as above was put in a cellophane tube and dialyzed against running tap water overnight. Through this procedure, the volume of the solution rose to 4,500 ml., but the inactivation of the enzyme was almost negligible provided that there were small quantities of calcium ion. This dialyzed enzyme solution was used in the following resin column treatment.

Resin Column Treatment—About 180 ml. of Kaken C-1 (SP-3) resin, 60–80 mesh at swelled H-form, was suspended in 1,500 ml. of *M*/15 sodium phosphate buffer solution (pH 6.5) and the pH of the suspension was maintained at this pH level (pH 6.5) for 1 hour with occasional addition of 2 *N* sodium hydroxide solution. After setting overnight, the resin was transferred into a column, 2.6 cm. in diameter, and then washed slowly by passing about 3,000 ml. of the same buffer solution.

Through this column, 4,500 ml. of the dialyzed enzyme solution was passed at the rate of 5–6 ml. per minute below 10°. In this process, almost all protease was completely adsorbed on the resin column, whereas the greater portion of coloring matter and proteinaceous impurities passed through.

The column was then washed with about 1,800 ml. of distilled water. By this washing, a remarkable amount of coloring matter was eluted together with proteinaceous impurity, but the loss of protease was practically negligible.

Then, the enzyme adsorbed on the resin column was eluted by passing about 2,700 ml. of Clark-Lubs' *M*/2 sodium borate buffer (pH 9.2) containing *M*/2 KCl at the rate of 5–6 ml. per minute. The effluent was divided into each 60 ml. of fraction and assayed. The appearance of elution process is shown in Fig. 3.

Then, these fractions were collected again into two large groups, *i.e.*, fraction I — about 900 ml. of enzyme-rich solution and fraction II — about 1,800 ml. of the remains. In fraction I, about 46 per cent of the adsorbed protease was recovered and the purity of this preparation was about thrice that of dialyzed enzyme solution, namely, about ten-fold that of original culture broth.

Crystallization—Crystallization of the protease from the above fraction I was carried out as follows: The protease-rich fraction prepared with the resin column treatment

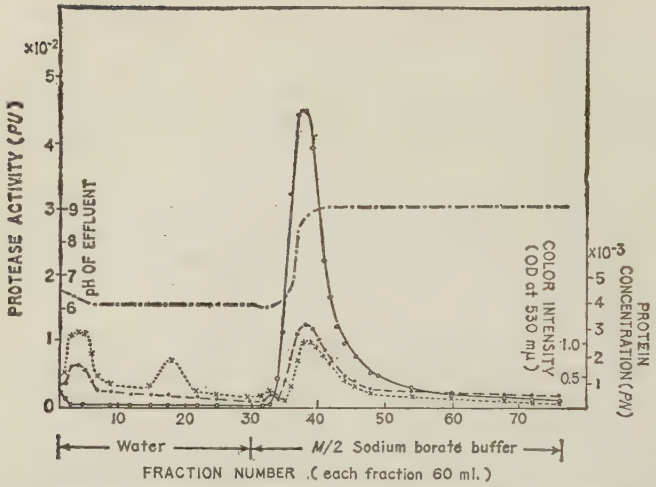


FIG. 3. Elution process for *Streptomyces griseus* protease adsorbed on Kaken C-1 (SP-3) resin.

Resin column: 2.6×33 cm., 180 ml. of resin (60–80 mesh at swelled H-form), buffered at pH 6.5 with *M*/15 sodium phosphate buffer. Protease adsorbed: 37.8 *PU*. Washing: 1,800 ml. of distilled water, 5–6 ml./minute. Elution: 2,700 ml. of 0.5 *M* sodium borate buffer (pH 9.2) containing 0.5 *M* KCl, 5–6 ml./minute.

—○— protease activity, —●— protein concentration, --×-- color intensity, —·— pH.

(fraction I) was neutralized with acetic acid and mixed with ammonium sulfate (40 g. per 100 ml. of enzyme solution) with vigorous stirring and then the mixture was kept at 4° for 2 hours or more. The resulting protease precipitate was collected by filtration on a small Buchner funnel with aid of small quantities of Hyflo Super Cel, and then was washed with saturated ammonium sulfate solution. In the filtrate, a larger amount of colored impurities were included, but enzyme was scarecely detected. The washed protease precipitate was dissolved in a small amount of 0.02 *M* calcium acetate solution and the remaining Hyflo Super Cel was removed by filtration.

This enzyme solution contained about 80 per cent of protease activity of the original enzyme-rich effluent, and the purity of preparation slightly increased. Then, the above enzyme solution was dialyzed against a large amount of 0.02 *M* calcium acetate solution at 4° overnight. The loss of enzyme activity was negligible in this procedure.

When there still remained a considerable amount of coloring matter in the dialysate, it seemed to be convenient to treat the dialysate with active carbon for eliminating the colored impurities. In this case, about 1–2 per cent (weight) of active carbon prepared from wood was mixed into the dialysate with strong stirring for 10–20 minutes at room temperature, and then the carbon was centrifuged off at 8,000×*g* for 20 minutes. By this process, almost all of the coloring matter were adsorbed on active carbon and almost colorless enzyme solution was obtained without any appreciable loss of protease activity.

Then, the enzyme solution prepared as above was slowly mixed with 1 volume of cold 96 per cent acetone at 5–10°, and the resulting precipitate containing impure proteinaceous substances other than enzyme was removed off by centrifugation. The enzyme, entirely remaining in the supernatant, was then completely precipitated by the further

addition of 2 volumes of cold 96 per cent acetone at 5–10° and was collected by centrifugation at 3,000×*g* after setting the mixture for several hours in a refrigerator.

This enzyme precipitate was thoroughly dissolved in a minimum volume of 0.01 *M* calcium acetate solution, and a slight amount of insoluble matter, when it still remained, was removed by centrifugation. To this concentrated protease solution, a small amount of cold 96 per cent acetone was added drop by drop with slow stirring at 5–10° until a very slight cloudiness scarcely appeared, and then the mixture was kept in a tightly stoppered tube in a refrigerator. Setting this mixture for several days with occasional addition of 1–2 drops of cold acetone gave rise to the gradual formation of small needle crystals of protease (Fig. 4).

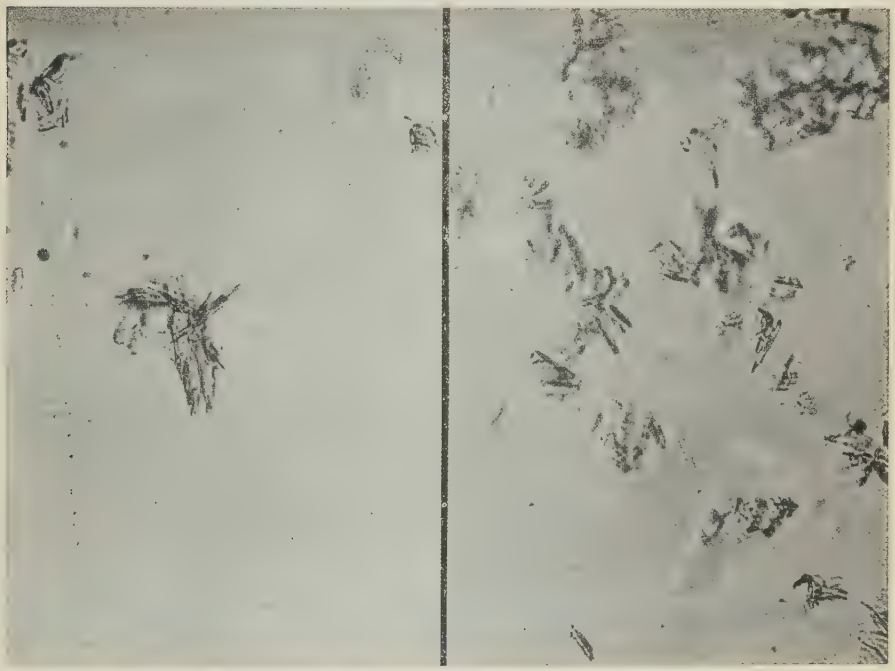


FIG. 4. Crystalline protease of *Streptomyces griseus*. (×600)

RESULTS

Preparation of the Enzyme Solution from the Culture Broth—Since there were abundant amounts of mycelia and muddy materials in the usual culture broth, it was hard to remove them by usual filtration. Though they were removable by centrifugation over 3,000×*g*, still a considerable amount of turbid impurity remained in the supernatant and contaminated the enzyme precipitate prepared by the subsequent salting out procedure. Therefore, further clarification procedure was necessary to remove the turbid inpurity.

It was found that the method for adsorbing the turbid matter by treating with calcium phosphate gel was the most successful for obtaining a clear enzyme solution. The procedure was accomplished as described in

the Methods.

By this treatment, a yellowish clear solution was obtained including about 80–90 per cent of protease activity and almost all of streptomycin in it, but the specific activity (purity; *PU/PN* or *PU*/mg. N) of this preparation was not increased. An attempt to reduce the concentration of calcium-ion to be added to the original solution resulted in an increase of turbidity in the resulting supernatant.

This method also can be applied in the clarification of the intact culture broth. A clear enzyme solution was also obtainable and the yields of protease and streptomycin were equal to those obtained in the former case.

When the culture broth was kept at 25–35° for 24 hours or more, the mycelia rose up to the surface of the broth as a result of some fermentation phenomena occurring gradually in the broth. After scraping out the majority of the floating cakes of mycelia, the remains were easily filtered off with aid of small amount of Hyflo Super Cel and then a yellowish slightly cloudy filtrate was obtained. Further clearing was performed equally as above by calcium phosphate gel method. In this case, the resulting enzyme solution was the clearest of all, though the yield of protease was reduced to about 60–70 per cent.

The removal of muddy impurity by adding lead acetate was also useful. The most suitable concentration of lead acetate to be added to the supernatant of broth seemed to be about 0.01 *M*. The experiment was performed at pH 5.6–6.0. By this method, about 80–90 per cent of protease and almost all of streptomycin was found in the resulting clear solution.

An attempt to remove the muddy impurity by use of zinc acetate was proved to be of no use. It was also an ineffectual trial to remove the muddy impurity with aluminium hydroxide gel.

Salting Out Procedure (*Separation of Protease and Streptomycin*)—It was proved that the addition of ammonium sulfate or sodium sulfate to the enzyme solution prepared by the calcium phosphate gel method or lead acetate method gave rise to the precipitation of enzyme leaving streptomycin in the supernatant. In this precipitate, about 90 per cent of protease activity was detected. On the other hand, almost all of streptomycin was discovered in the supernatant. Therefore, this procedure seemed to be useful for the separation of protease from streptomycin.

The necessary amount of ammonium sulfate to precipitate the protease seemed to be dependent upon the concentration of proteinaceous materials including protease and other impurities. That is, when the enzyme solution was prepared immediately from culture broth by centrifugation, the necessary amount of ammonium sulfate was about 40 g. per 100 ml. of enzyme solution and 95 per cent of original protease activity was obtained in the salting out precipitate. However, if the enzyme solution prepared with calcium phosphate gel method or lead acetate method was employed, a larger amount of ammonium sulfate, more than 60 g. per 100 ml. of enzyme solution, was needed to get a high yield of protease. Since the temperature of

treatment exerted a great influence upon the formation of precipitate, the salting out procedure should be performed below 10°. Although sodium sulfate was also useful for this procedure, sodium chloride and magnesium sulfate was not.

Acetone precipitation method was not successful, because both protease and streptomycin were precipitated simultaneously by this reagent. Methanol was also undesirable for this purpose because of its lower ability to separate enzyme from streptomycin, and of its ability to inactivate the protease.

Resin Column Treatment—Considering the data from the screening test together with information on the stability of this enzyme, Kaken C-1 (SP-3) resin was mainly adopted throughout this work. Using this resin, the purification of the protease was successfully accomplished as described in the Methods. Through this treatment, highly purified enzyme solution was obtainable with 40–70 per cent recovery of the adsorbed enzyme.

The necessary amount of resin is not only determined by the quantity of enzyme in the passing solution but also by the concentration of other proteinaceous impurities which compete with the enzyme in adsorbing on to resin. Threfore, the higher the purity of the passing enzyme solution, the larger the quantity of enzyme adsorbed on resin. In the present case, the suitable amount of resin seems to be about 5 ml. (at swelled H-formed) per 1 *PU* of the protease.

Though it is possible to adsorb all the protease on the resin by reducing the amount of protease in passing solution, the efficiency of eluting protease was considerably reduced in such a case. Therefore, it seemed to be better to charge a slightly excess amount of protease than that comparable to the resin capacity.

In case of elution process, it was possible to use the other alkaline buffer containing high concentration of salts instead of above sodium borate buffer.

In another experiment using Duolite C-10 resin, the similar results were observed as in the case of Kaken C-1 (SP-3) resin.

Crystallization—Protease existing in the above enzyme-rich effluent was conveniently concentrated and purified by precipitation with ammonium sulfate and with acetone. Then it was crystallized from acetone solution.

However, the crystallization of this protease seemed to be fairly difficult compared to that of other proteases, and occasionally the amorphous form of this enzyme was also found to be mixed with the crystalline preparation. Crystallization of this enzyme with ammonium sulfate was unsuccessful.

Representative results on the purification of the protease are summarized in Table V. The whole purification procedures are also shown in Scheme 1 in which the mean value on the yield and purity of the protease in each step are noted together.

Some Properties of Streptomyces Protease G—The crystalline protease was a colorless fine needle and showed a specific activity of 0.12 *PU*/mg.N. By paper electrophoresis, this preparation was observed to have single

TABLE V

A Representative Result on Purification of Streptomyces griseus Protease

Fraction	Volume (*ml.*)	Protease			Purity (*PU/PN*)	Streptomycin		Color 530 mμ (%)
		Conc. (*PU/ml.*) $\times 10^{-3}$	Total (*PU*)	Yield (%)		Total (*unit*) $\times 10^{-5}$	Yield (%)	
Supernatant of culture broth	9,000	7.40	69.00	100	0.88	216	100	100
Solution prepared by Ca-phosphate method	10,500	5.66	59.43	86.1	0.83	197	91.2	34
Solution of salting out precipitate	3,400	15.67	53.28	77.2	3.10	7	3.2	22
Supernatant of salting out	12,500	0.10	1.25	1.8	0.03	190	87.9	14
Dialyzed solution before charged	4,500	11.40	51.30	74.4	4.80	—	—	16
Effluent solution passed through	4,500	3.00	13.50	19.6	2.10	—	—	7
Apparent amount of adsorbed enzyme	—	—	37.80	54.8	10.10	—	—	9
Washings with water	1,800	0.31	0.56	0.8	0.56	—	—	2
Eluate with borate buffer (I)	880	19.74	17.37	25.2	11.21	—	—	1
Eluate with borate buffer (II)	1,820	3.24	5.90	8.6	6.04	—	—	1
Dialyzed solution of salting out precipitate from fraction (I)	260	53.65	13.95	20.2	12.88	—	—	—
Solution of acetone precipitate	35	297.40	10.41	15.1	16.80	—	—	—
Solution of crystals	20	262.50	5.25	7.6	22.00	—	—	—

electrophoretic component. It was fairly stable over the pH range of 5–9 and showed maximal activity at pH 7-8 when measured by the Folin color method. It was easily soluble in water, dilute salt solution or diluted organic solvents, but not in acetone or ethanol solutions above 60 per cent. It also appears to be worthy of note that this enzyme is specifically stabilized by the presence of small amounts of caclium ion. Concerning these properties of this enzyme, further detailed information will be presented in the next papers.

DISCUSSION

Protease of *Streptomyces griseus* could be separated from the culture broth and purified by the foregoing method. However, it appears to be unsuitable

SCHEME 1

Summary of Purification Procedures for Streptomyces griseus Protease

	Yield of *PU* (%)	Purity (*PU*/*PN*)
Supernatant of culture broth	100	1
added Na_2HPO_4 and $CaCl_2$, centrifuged		
Precipitate (*discarded*) / Supernatant	80-90	1
salted out by $(NH_4)_2SO_4$, centrifuged		
Supernatant (streptomycin fraction) / Precipitate	70-80	3-4
dissolved in water, centrifuged, dialyzed		
Precipitate (*discarded*) / Dialyzed solution	70-80	3-5
passed through Kaken C-1 (SP-3) resin column		
Effluent (*discarded*) / Protease adsorbed on resin	50-70	—
washed with water, eluted with borate buffer		
Washings (*discarded*) / Eluate (rich fraction)	25-40	11-13
salted out by $(NH_4)_2SO_4$, filtrated		
Filtrate (*discarded*) / Precipitate	20-35	12-14
dissolved in dil. $Ca(CH_3COO)_2$, dialyzed		
Dialyzed solution	20-35	12-14
added acetone, centrifuged		
Supernatant (*discarded*) / Precipitate (amorphous protease)	10-15	16-18
dissolved in dil. $Ca(CH_3COO)_2$, added acetone until cloudy, kept at 4°		
Crude crystalline protease	5-10	22-24

for industrial application to separate the protease by means of salting out, because the existence of high concentration of ammonium sulfate in the resulting streptomycin fraction brings about a difficulty in the purification of streptomycin. Therefore, further improvement should be carried out on this subject and this approach is currently being examined by the authors.

As mentioned above, crystallization of this enzyme appeared to be fairly

difficult and the resulting crystalline preparation occasionally includes amorphous form. This may be due to the similar solubilities of both forms in acetone. More data are needed on this point and the procedure on this step is still under study by the authors. However, since this preparation appeared to be enzymatically homogeneous, the investigation on the enzymatic properties of this enzyme has been conducted by use of this preparation.

In above experiment, cation exchange resin such as Kaken C-1 (SP-3) has been mainly used for resin column treatment. But, another type of resin lacking ion exchange faculty, *e.g.* Kaken Phenol resin, was also proved to be useful for such a purpose. Undoutedly, it appears that the enzyme molecules might be adsorbed on undissociated hydroxyl group of the latter resin by the effect of some attracting power other than ion exchange phenomenon. Indeed, it was confirmed by Yanagita *et al.** that the substitution of hydroxyl group for methoxyl group resulted in the cancellation of adsorption capacity for protease.

This information is worthy-wise as suggesting the mechanism of adsorption power acting between resin and enzyme.

SUMMARY

1. It was found by the authors that *Streptomyces griseus*, a typical strain used for the industrial production of streptomycin, produced a remarkable amount of protease in usual culture broth together with streptomycin.

This enzyme was separated from streptomycin and highly purified by a convenient method as follows: A filtrate of the broth was mixed with Na_2HPO_4 and $CaCl_2$ and the resulting calcium phosphate gel was removed together with turbid impurities. From this clear solution, the enzyme was precipitated by salting out leaving streptomycin in the supernatant. After dissolving and dialyzing this enzyme precipitate, the resulting enzyme solution was passed through a column of Kaken C-1 (SP-3) resin buffered at neutral pH and then the adsorbed enzyme was eluted with alkaline buffer solution containing high concentration of salts. From this eluate, the enzyme was concentrated and purified by salting out and acetone precipitation, and crystallized as needles from acetone solution.

2. In resin column treatment, a few of other kinds of resins such as Duolite C-10, S-30 or Kaken Phenol-type resin was also proved to be useful. On the basis of this experiment, the mechanism of adsorption between resin and enzyme was discussed.

3. It was proved by paper electrophoresis that the crystalline preparation of this enzyme was composed of a single electrophoretic component. This enzyme was fairly stable over the pH range from 5 to 9, and its maximal activity was appeared at pH 7–8.

The authors wish to express their thanks to Dr. T. Akahira, Dr. M. Yanagita,

* Yanagita, M., Kawabe, H., Nomoto, M., and Watanabe, M., the General Meeting of the Japanese Biochemical Society, November (1955).

Prof. K. Sakaguchi, Prof. K. Okunuki and Dr. B. Hagihara for their kind guidances and useful suggestions. The authors are also grateful to Mr. S. Fujita for preparation of the enzyme and to Mr. H. Kawabe for resins, besides their helpful advices during the course of this work. Thanks are also due to the Kaken Chemical Co., Ltd. for the continual supply of culture broth.

The present work was aided in part by the grant of Scientific Research Fund of the Ministry of Education for which the authors wish to thank.

REFERENCES

(*1*) Hagihara, B., *Ann. Rep. Sci. Works, Osaka Univ.*, **2**, 35 (1954)
(*2*) Anson, M. L., *J. Gen. Physiol.*, **22**, 79 (1938)
(*3*) Kunitz, M., *J. Gen. Physiol.*, **30**, 291 (1947)
(*4*) Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 624 (1927)
(*5*) Egami, F., Hyojun Seikagaku Jikken, Kobundo Co. Ltd., Tokyo, p. 232 (1953)
(*6*) Hagihara, B., Matsubara, H., Nakai, M., and Okunuki, K., *J. Biochem.*, **45**, 185 (1958)
(*7*) Kawabe, H., and Yanagita, M., *J. Sci. Res. Inst.* (*in Japanese*), **28**, 264 (1952)
(*8*) Okunuki, K., Hagihara, B., and Ukita, T., *Japan Pat.* in application (1957)